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The Use of Cell Colonies on Glass for Evaluating Nutrition and Growth in Roller-Tube Cultures [1,2]

ROBERT L. EHRMANN [3] *and* GEORGE O. GEY,
Division for Cell Physiology, Department of Surgery, The Johns Hopkins Hospital and Medical School, Baltimore, Md.

In the study of factors influencing cell growth, there is a distinct need for an easily grown standard strain of cells which produces well-formed colonies in fluid medium without a plasma clot. Although the cultivation of cells in fluid medium is not a new technique (*1–3*), its value in cell-colony experiments has been unrecognized. The elimination of plasma clot from cell-colony cultures removes a source of confusion in evaluating response to nutritional factors, and greatly simplifies the cultural procedures. A strain of cells filling the above need is described below, together with a simple technique of setting up and selecting replicate groups of cultures.

The cells studied were normal rat fibroblasts, strain *14pf*, which have been under continuous cultivation in this laboratory since 1938 (*4*). A remarkable characteristic of these cells is their growth as a monocellular layer on the glass wall of a roller tube. This growth in one plane makes colony-area measurement a far more reliable growth index than in other cell strains where colonies increase in thickness as well as in area. Being only one cell thick, the colonies are barely visible to the naked eye, but are clearly seen through the microscope with the iris diaphragm closed down. When a culture is started with 20 or 30 fragments, and the colonies

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[3] Senior Assistant Surgeon, U. S. Public Health Service.
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allowed to grow for 2 or 3 weeks, the tube wall becomes completely coated with a monocellular membrane (fig. 1) which is readily transferred. Fewer fragments would require a longer growth period to coat the tube. The membrane is easily pushed down to the bottom of the tube, removed to a Petri dish, heaped together into a compact mass, and cut into 100 or more fragments, each measuring 1 to 2 mm. in diameter. These fragments may then be used for setting up a large number of experimental tubes.

Cultural Technique

Maintenance of Stock Cultures

Maintenance of stock cultures is not time-consuming in comparison with other procedures for continuous cultivation. With colonies grown directly on the glass wall, nutrient fluid is renewed twice a week, and transfers to new tubes made every 4 weeks. Transfers could be made much more frequently, but they are delayed in order to let the membrane thicken through cellular crowding, and thus yield a maximum amount of tissue for experimental procedures. Moreover, a heavy membrane is easier to remove en masse than a light membrane, in which the cells are more firmly attached to the glass and less adherent to one another. If a new group of 4 cultures is set up once a week for 4 successive weeks, every week from then on, one of the 4 groups will be ready for transfer. Thus, every week 4 heavily coated tubes will become available for setting up experiments and for transfer to 4 more stock tubes. The maintenance of these 16 stock cultures requires only 2 to 4 hours a week for cultural procedures. Roller-tube cultures of strain *14pf* rat fibroblasts have been actively growing in fluid medium in this laboratory for almost 2 years. To maintain the strain when no immediate experiments are planned, reduction of the maintenance temperature to 31° C. and the number of fragments to 4 or less per tube, reduces treatment to once per month, or less (*4*). The culture medium consists of 4 parts balanced salt solution (Gey), 1 part 50% beef-embryo extract, and 5 parts human placental-cord serum. This type of serum has been uniformly satisfactory, whereas batches of horse, ox, and chicken serum tested to date on this strain of cells have had an inhibitory effect. (In a personal communication, Dr. R. C. Parker states that *14pf* in his laboratory grows well in 40 percent horse serum and chick-embryo extract.) Beef-embryo extract is used because it is easily prepared in bulk quantity. Chick-embryo extract [4] has also been tried and found to be satisfactory. Each roller-tube culture receives 10 drops of the mixed medium.

Transfers are made by freeing the cellular membrane, which is almost invisible even in a heavily coated tube. This is accomplished with a sterile, curved-tip, Gey-type pipette fitted with a ¼-ounce rubber Asepto bulb, which has been in general use for many years in roller-tube work. With the tube held at a slant, a small amount of the old fluid medium is aspirated. The fluid-filled tip of the pipette is then touched to the wall of the tube above the cell-covered portion, and fluid allowed to flow down over the free border of the membrane. This washing procedure begins to loosen the free border from the glass and causes it to become visible by contraction and thickening. The process is aided by gently nudging the retracting border with the pipette tip, while rotating the tube slowly so as to expose all parts of the membrane to the nudging and washing. When the pipette is empty, more fluid is drawn up, and the process continued. When the membrane has been pushed in this manner to the bottom of the tube, it floats freely as a crumpled white mass in the fluid medium. It is drawn up into the pipette and transferred, together with 4 or 5 drops of medium, to a sterile Petri dish. With a pair of flame-sterilized No. 11 Bard-Parker knives, each on a No. 9 handle, the tissue mass is drawn away from the puddle of medium, heaped together into a little ball, and cut up into fragments about 1 mm. in diameter. Ten

[4] Sample kindly submitted by Microbiological Associates, Inc., Bethesda, Md.

drops of fresh medium are placed in a dry, sterile roller tube, and the inner surface made moist by rotating the tube in a nearly horizontal position while spreading the fluid throughout the lower two-thirds with the back of the curved-tip pipette. When this is accomplished, 20 to 30 of the newly cut fragments are drawn up into the pipette, and distributed evenly over the lower half of the moist tube wall. The tube is left standing upright in the incubator for about half an hour to allow the fragments to become adherent to the wall, and then is placed in the roller drum for incubation.

Microscopic examination after the first hour of incubation shows early signs of cellular activity. Here and there around the periphery of each fragment, membranous processes extend outward from individual cells. By the second hour, migration has started. Mitoses are seen within 24 hours, and, after a week of growth, the colony diameters may measure 10 mm. At variable times, peripheral cells break off and become scattered over the glass between the growing colonies. Through proliferation of these scattered cells and continued growth of the colonies, the inner surface of the tube becomes coated with a monocellular membrane. The remarkable ability of the cells to form such a membrane makes it possible to count almost every cell in it, including those surrounding the central fragments (fig. 1). After 2 to 3 weeks' growth, only traces of the original transferred fragments can be found, due to cellular migration, and the membrane may by this time completely coat the inner surface of the tube. With continued growth, the membrane of cells becomes thicker through crowding, but not through overlapping of cells. After 4 weeks, when the tube wall is heavily coated with the membrane, enough tissue is available for setting up 20 or more experimental tubes.

Preparation of Cultures for Quantitative Experiments

To set up an experiment from a stock culture, the membrane is removed and cut into fragments as described above. With the fragments sitting in a puddle of old medium, small droplets of 10 percent cord serum in balanced salt solution are placed near-by, the number of droplets equal to the number of cultures to be set up. With a pair of knives, a fragment is drawn to the edge of the puddle, lifted out on a knife point, and placed in one of the droplets. This is repeated until there are 4 fragments in each droplet. Each droplet will supply the tissue for a single experimental tube. An effort is made to select equal-sized fragments for any one droplet so that the 4 colonies growing from them will have approximately equal areas. The simplest procedure in selecting fragments is to start with the largest and, as they get used up, continue with the next largest, and so on until each droplet in turn has been supplied with its quota of fragments. The result is a series of droplets, each containing 4 matched fragments whose average size diminishes from the first to the last droplet. Ten drops (approximately 0.6 ml.) of 10 percent cord serum are placed in a sterile roller tube, and spread over the inner surface as already described. A small amount of this fluid is drawn into the curved-tip pipette. The pipette tip is then touched to the glass alongside one of the fragment-containing droplets, and enough fluid discharged from the pipette into the droplet to stir up the fragments in case they are adherent to the Petri dish bottom. Immediately thereafter, the fluid is drawn back again into the pipette, together with the fragments. The pipette is reinserted into the roller tube, and the 4 fragments discharged one by one in a row along the moist surface. In order to have the fragment locations the same in each tube, guide marks are placed on the outside with a wax pencil. After standing the tubes upright as before to allow adherence of the fragments, they are placed in the roller drum. On completion of a 48-hour incubation, the cultures are classified into replicate groups

on the basis of area measurements, to be described, and then used in a planned experiment.

Evaluation of the Method Used in Experiments With Strain *14pf*

Experiments performed with the *14pf* strain of rat fibroblasts grown on glass have a special value in revealing the early responses of cells to their nutritional environment. Due to the monocellular type of growth and the absence of clot, every cell in the outgrowth is in direct contact with the experimental fluid. If the fluid is nutritionally deficient, inhibitory, or growth-stimulating, these conditions may produce a noticeable effect within 72 hours. It should be emphasized that these early responses do not necessarily indicate how the strain would behave under prolonged cultivation in a given experimental medium. Yet, much valuable information can be gained from studying the early responses of cells to their chemical environment, and the *14pf* strain of normal rat fibroblasts is particularly well suited for this purpose.

In planning an experiment, the question always arises whether to start growing the fragments directly in the experimental media, or whether to add the media after the colonies have had a preliminary growth period. The choice of method depends on the problem being investigated. The first method is best when one is studying specifically the factors that influence outgrowth of cells from a newly transplanted fragment. If a medium will not support the outgrowth of cells from a fragment, the second method allows the cytologic effects of this medium to be studied more easily. The outstanding feature of the second method, however, is that it permits an accurate classification of cultures into replicate groups on the basis of area measurements. The preliminary growth period lasts 48 hours and is carried out in 10 percent cord serum diluted with balanced salt solution. The retardation of growth resulting from this marked serum dilution produces small compact colonies whose areas are easily measured (fig. 2). Moreover, it is believed that this high dilution of nutrients renders the cells more susceptible to subsequent growth-promoting or inhibiting substances.

Colony area is one of the chief criteria used for estimating experimental effect. It is easily and rapidly measured with the aid of an ocular micrometer, and reflects changes in the rates of migration, mitosis, and death of cells. Which of these plays the dominant role in altering the area of a colony can be determined by direct microscopic observation. Dividing cells stand out in bold relief against the pale membrane of resting cells, making mitotic counts in the living roller-tube colony quite feasible.

Experiments

Effect of Embryo Extract

These experiments tested the possibility of using *14pf* colonies on glass for the bioassay of growth-promoting substances. Embryo extract was selected as the test substance, since its growth-promoting property has been known for many years. The experiments were designed to see if

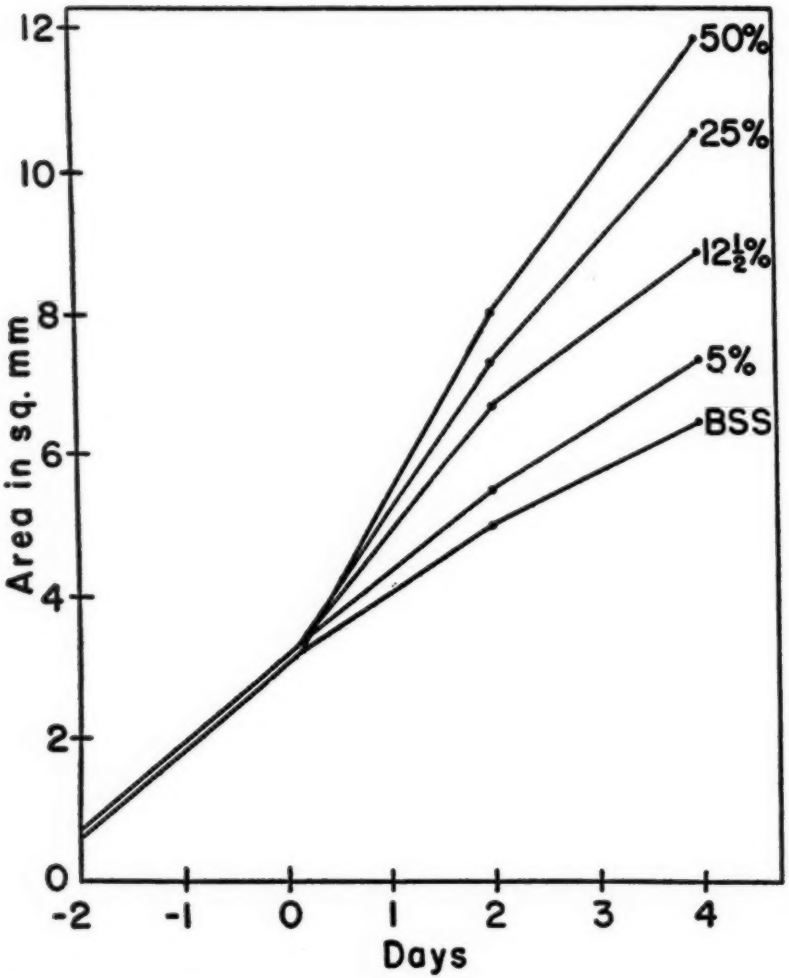
differences in growth stimulation could be obtained from corresponding differences in concentration of beef-embryo extract. Such an effect was found in a series of six experiments. In all experiments, the tubes were set up with four fragments each, cultivated as already described for 48 hours in 0.6 ml. of 10 percent cord serum, and then classified (by the procedure explained in the next section) into replicate groups on the basis of area measurements. The number of tubes per group was varied from experiment to experiment, until 6 tubes appeared optimal. After classification, each group received a different concentration of embryo extract. The concentrations were prepared beforehand by diluting the extract with different amounts of balanced salt solution. In the first five experiments, 0.1 ml. of the appropriate dilution was added to each tube within a group without removal of the old fluid, and after incubation for an additional 3 to 5 days, the colony areas were again measured. Such an experiment is illustrated by text-figure 1.

A clearer concept of the effect may be obtained from text-figure 2, in which the final 4-day areas in this same experiment have been plotted against the corresponding embryo-extract concentrations. The steep part of the curve represents the concentration range in which the greatest differential response was obtained. This suggests the advisability of diluting unknowns to an optimal level before testing.

In the last experiment of this series, instead of adding 0.1 ml. of diluted embryo extract to the old medium in each tube, the 10 percent serum in each tube was removed and replaced by 0.5 ml. of a mixture of 4 parts balanced salt solution, 5 parts cord serum, and 1 part diluted embryo extract. The dilutions differed for each experimental group of cultures as before, and a clear-cut differential response was again obtained. By this increase in cord-serum concentration from 10 percent to 50 percent, the cells were provided with better nutrition which allowed observations to be made over a longer period of time. However, when later work showed that cord serum at 50 percent sometimes has an inhibiting effect, a concentration of 25 percent or 30 percent was selected as optimal for subsequent experiments.

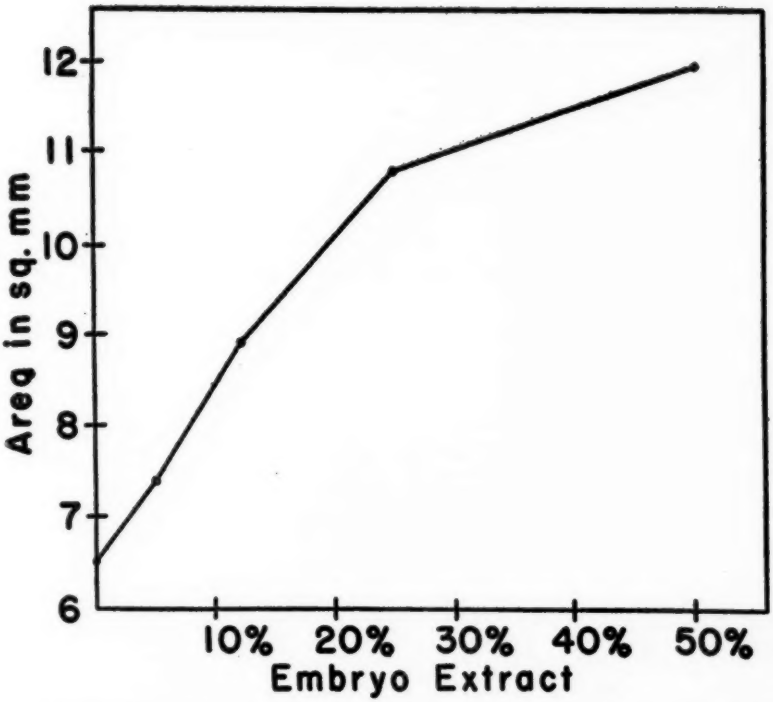
Effect of Hemoglobin

The usefulness of the experimental technique for *14pf* on glass was tested further in a study of the effect of hemolyzed serum on the growth of cells. Preliminary experiments showed that slightly hemolyzed serum produced definite stimulation of colony outgrowth and mitosis during a 7-day observation period. To find the source of this stimulation, specimens of hemoglobin were prepared from the blood of 10 different rats by crystallizing three times from distilled water. Each of the 10 specimens was then added to 25 percent cord serum to give a final concentration of 200 mg. percent, thus providing 10 different hemoglobin solutions. The experiments were started in the usual manner on classified groups of 48-hour cultures, 6 tubes per group. One group was used for testing each hemoglobin solution. Any single experiment contained several hemo-



TEXT-FIGURE 1.—Differential response of *14pf* to various concentrations of beef-embryo extract. The percentages refer to the concentration of embryo extract before addition to the cultures. Each curve represents the average colony area for a group of 5 roller-tube cultures, except the 12½% percent curve which represents 4 tubes. Days −2 and −1 indicate the preliminary growth period in 10 percent cord serum diluted with balanced salt solution. BSS stands for balanced salt solution.

globin groups, and one or more groups to serve as controls. After forming the groups, the 10 percent serum in each experimental tube was replaced by 0.5 ml. of the appropriate hemoglobin solution, and in each control tube by 0.5 ml. of 25 percent cord serum alone. Each experiment was performed on a different date. Text-figure 3 shows the experimental

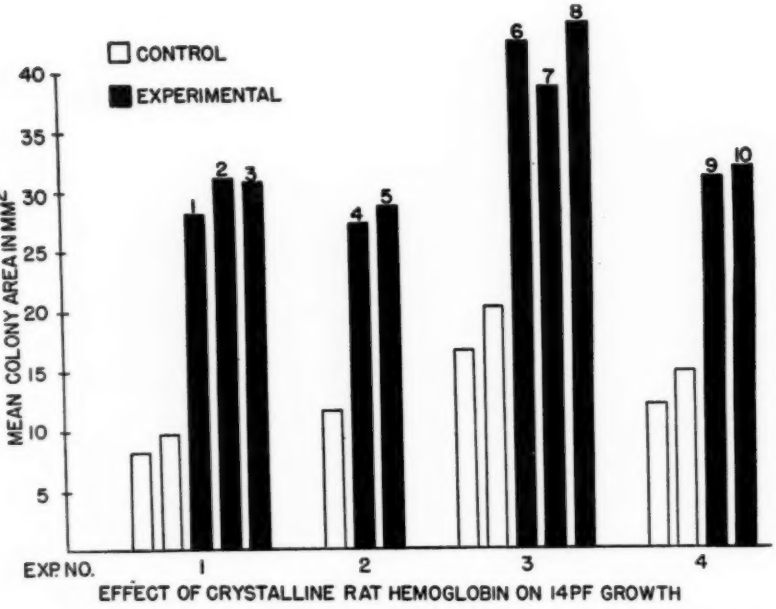


TEXT-FIGURE 2.—Same as text-figure 1, with the final average colony areas plotted against the corresponding embryo-extract concentrations.

results after 7 days. Marked growth stimulation resulted from each hemoglobin solution. The effect is also strikingly displayed by a photograph of the fixed and stained cultures from an experiment testing 1 of the 10 hemoglobin solutions (fig. 3). The finding of growth stimulation by hemoglobin may be related to a similar observation by Baker (*5*) who incorporated hemolyzed rat blood cells, or crystalline rat hemoglobin at a concentration which we have estimated to be 11.9 mg. percent in a culture medium incomplete for continuous growth, containing casein digest, glycine, nucleic acid, and glutathione. The hemoglobin used by Baker caused definite growth stimulation of normal and malignant rat fibroblasts.

STUDIES ON THE ULTRAFILTRATES OF HORSE SERUM AND CHICK-EMBRYO EXTRACT

A large part of the growing interest in fractionated biological fluids has been in the use of serum ultrafiltrate (*6*) or, more recently, embryo-extract ultrafiltrate (*7*), as media components for long- or short-term cultivation of cells. Since the nutritional role of these ultrafiltrates needs further clarification, they were tested by the roller-tube method already

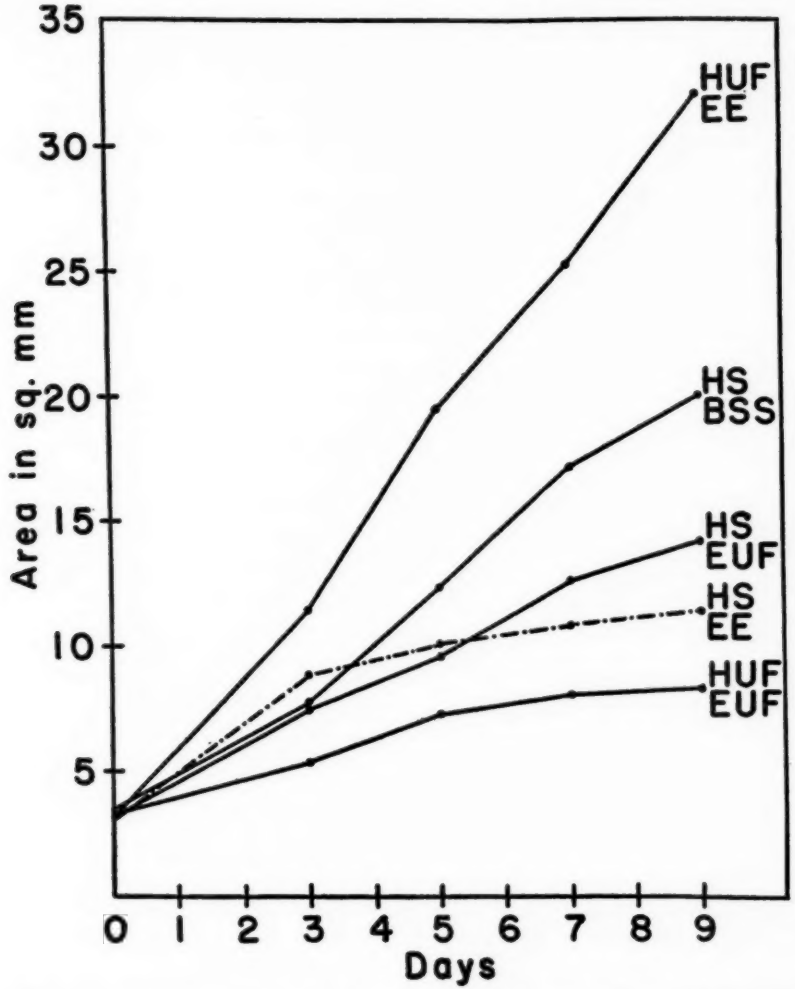


TEXT-FIGURE 3.—Effect of crystalline rat hemoglobin at 200 mg. percent on *14pf* growth. Each bar represents the average colony area for a group of 6 roller-tube cultures, except experiment 2, with 5 tubes per group. The numbers over the bars indicate the particular rats which provided the hemoglobin samples. In experiment 4, the cultures were protected with 100 units penicillin G sodium (Merck) and 10 units dihydro-streptomycin (Merck) per ml.

described for glass-grown colonies of strain *14pf*. In these initial studies, samples of horse serum (HS), horse-serum ultrafiltrate (HUF), hyaluronidase-treated 50 percent chick-embryo extract (EE), and ultrafiltrate of this chick-embryo extract (EUF) were kindly given to us by Dr. W. R. Earle of the National Cancer Institute. These samples were prepared in his laboratory. Whether tested alone or in combination with other components, the concentration of horse serum or its ultrafiltrate was 30 percent, while that of embryo extract, or its ultrafiltrate, was 20 percent. Dilutions were made with balanced salt solution (BSS, Gey). After the initial 48-hour growth period, the 10 percent cord serum in each tube was replaced by 0.5 ml. of the medium to be tested.

In experiment 1, illustrated by text-figure 4, various combinations of these media components were tested, and their effects evaluated on the basis of colony area, frequency of mitoses, and cytologic appearance of the cells. Throughout the 9-day observation period, there was active growth in the control cultures containing 30 percent horse serum alone (HS+BSS), with a 6-fold increase in average colony area. These control cultures appeared in good condition during 9 days of observation, except for one tube in which large portions of the colonies showed cellular retrac-

tion and death on the ninth day. In the tubes containing chick-embryo extract, in addition to the diluted horse serum (HS+EE), the results were most unexpected. Although in the first 3 days the growth rate in these tubes with extract was slightly more rapid than in the horse-serum controls, the early appearance of diffusely scattered cellular degeneration suppressed growth from then on, so that by the ninth day, they showed only half as much growth as the controls. Since the experimental medium was nutritionally richer than the control medium, the early degeneration



TEXT-FIGURE 4.—Response of *14pf* to combinations of horse serum, chick-embryo extract, and their ultrafiltrates. Each curve represents the average colony area for a group of 6 roller-tube cultures except the control curve (HS+BSS), which represents 5 tubes.

and growth suppression in the experimental tubes indicate a deleterious effect of the medium, rather than nutritional inadequacy. These findings are in contrast to those of Sanford *et al.* (*7*), who tested similar combinations of media components on strain L mouse fibroblasts, using the method of nuclear counts for evaluation of effect (*8*). They found much more growth in the cultures containing 40 percent horse serum plus 20 percent chick-embryo extract, than in 40 percent horse serum alone. This shows how differently two strains of cells can behave when exposed to the same media components.

When horse-serum ultrafiltrate replaced whole serum, in combination with chick-embryo extract (HUF + EE), there was a rapid increase of area and an abundance of dividing cells (text-fig. 4). The stimulation, however, was accompanied by the appearance of breaks about the central fragments with rounding up and loss of cells from the margins of the breaks. These breaks coalesced, forming a hole in the center of each colony. With the continued loss of cells, the holes became larger until, after 5 days, they occupied the greater portion of each colony. From then on, the increase in hole-size kept pace with the rapidly proliferating cells in the outer portion of each colony, so that the area increase seen in text-figure 4 beyond 5 days reflects nothing more than the rapid expansion of cellular rings. The central holes are believed to result from a nutritional inadequacy of the medium, rather than from the action of deleterious substances, because such holes appear in colonies of many cell strains on failure to replenish the fluid medium, and because in this experiment the peripheral cells continued to multiply rapidly, and appear active and healthy. It has been our experience that cellular destruction replaces such peripheral activity when deleterious substances are responsible for growth inhibition. The nutritional inadequacy of horse-serum ultrafiltrate in combination with chick-embryo extract is further suggested by failure of *14pf* fragments to produce colonies when transplanted directly into this medium without the preliminary 48-hour cultivation in 10 percent cord serum. There was only abortive migration of a few cells. Sanford *et al.* (*7*), using the nuclear count method, also found this combination of components to be a poor medium when tested on strain L mouse fibroblasts. The fact that the nuclear count method and the technique for glass-grown colonies showed a nutritional deficiency manifested in different ways is of importance, and suggests that these two methods used together may provide a clearer concept of the nutritional effects of media upon cells. The former accurately measures the amount of over-all cellular proliferation, whereas the latter reveals and evaluates the multitude of individual cell responses which lead to the final result. Generalizations must be made cautiously, however, until a strain of cells is found that can be studied by both methods.

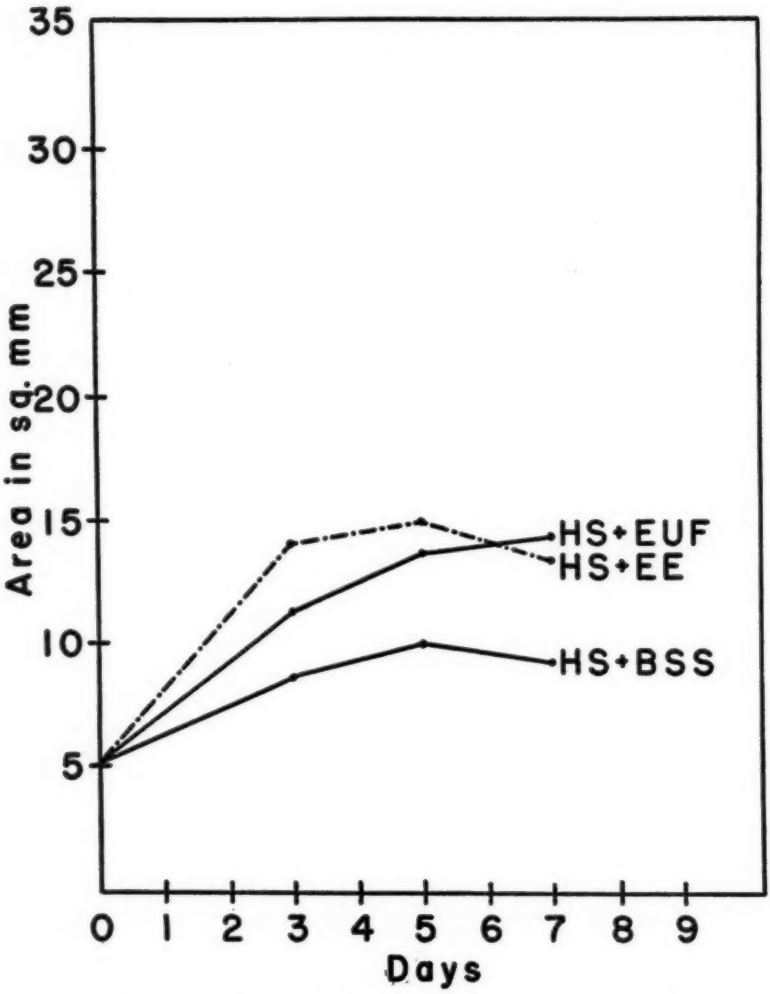
The remaining two curves in text-figure 4 show the effect of chick-embryo extract ultrafiltrate in combination with 30 percent horse serum (HS + EUF) or with 30 percent horse-serum ultrafiltrate (HUF + EUF). Chick-embryo extract ultrafiltrate, in combination with horse serum,

suppressed growth rate below the controls containing 30 percent horse serum alone (HS + BSS), but the suppression was less marked than that produced by the combination of whole chick-embryo extract with horse serum (HS + EE). With the two ultrafiltrates in combination (HUF + EUF), there was very little growth, but the colonies remained relatively free from degenerating cells throughout the 9-day observation period.

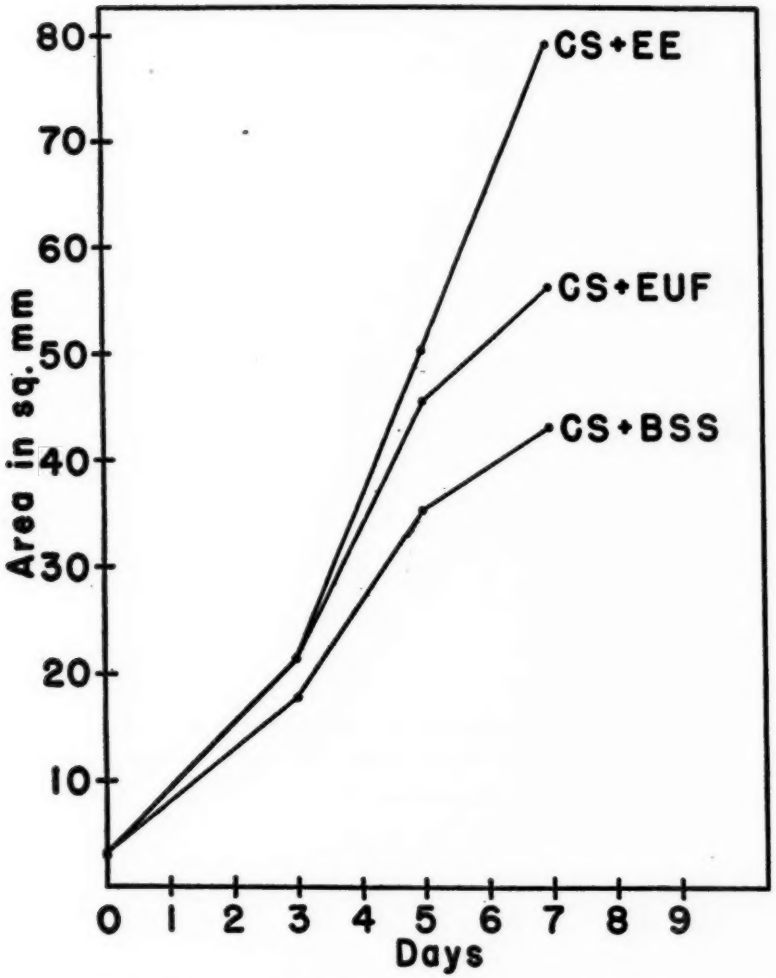
By comparing the rapid area increase due to chick-embryo extract in horse-serum ultrafiltrate (HUF + EE) with the inhibition from its combination with whole horse serum (HS + EE), one may deduce that substances responsible for inhibition were present in the large molecular fraction of the horse serum tested. Since inhibition resulted when this horse serum was combined with either chick-embryo extract or its ultrafiltrate, it was felt that dilution of these 3 media components might decrease the inhibitory substances to a less effective concentration. Therefore, in experiment 2, horse serum was used at 10 percent instead of 30 percent, and the chick-embryo extract and ultrafiltrate concentrations were reduced from 20 percent to 10 percent. Text-figure 5 summarizes the results of this second experiment using diluted media. All cultures showed dividing cells after 3 days, with relative growth stimulation in both experimental groups of tubes. Whole chick-embryo extract produced a greater effect than its ultrafiltrate. A statistical analysis proved the stimulation in both experimental groups to be highly significant, with P less than .01. Thus, the second experiment at 3 days supported the findings of Sanford *et al.* (7) that growth stimulation could be produced not only from chick-embryo extract, but also from its small molecular fractions obtained through ultrafiltration. Examination of the *14pf* experimental tubes beyond 3 days revealed the same interesting phenomenon observed in the first experiment. The initial growth stimulation in the tubes containing chick-embryo extract was replaced by growth suppression and large-scale death of cells. Disintegration of these cells in the peripheral parts of colonies caused an actual decrease of colony area, as seen from the 7-day measurements.

The problem of inhibition was eliminated when human placental-cord serum (30% in BSS) was substituted for horse serum. Thus, in a third experiment (text-fig. 6), growth stimulation from 20 percent chick-embryo extract ultrafiltrate (CS+EUF) was highly significant, with P far less than .01. The effect was not as great, however, as the stimulation from whole chick-embryo extract (CS+EE). All cultures appeared active and healthy throughout the 7-day test period.

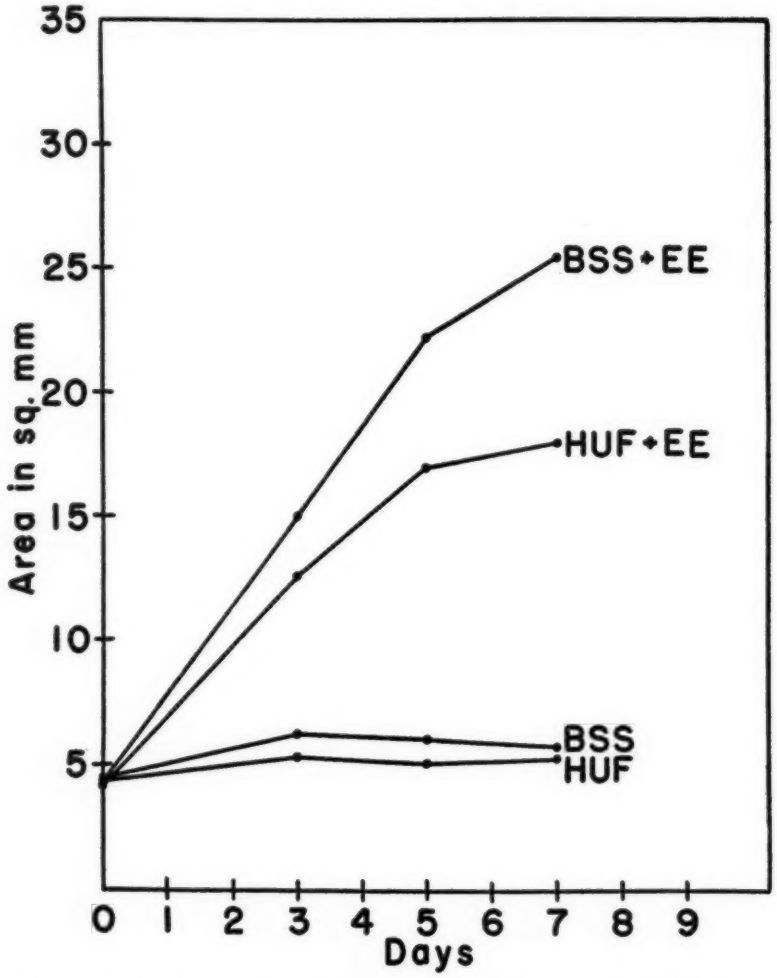
To evaluate the growth-promoting effect of horse-serum ultrafiltrate, it was compared with balanced salt solution. Replicate groups of cultures, each containing 5 tubes, were grown for 7 days either in 30 percent horse-serum ultrafiltrate or in balanced salt solution, with and without 20 percent chick-embryo extract. The results of this experiment are shown in text-figure 7. Marked growth stimulation occurred in both groups containing embryo extract, together with the same type of central-hole formation as was found in experiment 1. It will be seen from text-figure



TEXT-FIGURE 5.—Effect of dilution on response of *14pf* to combinations of horse serum with chick-embryo extract or its ultrafiltrate. Each curve represents the average colony area for a group of 6 roller-tube cultures.



TEXT-FIGURE 6.—Response of *14pf* to chick-embryo extract and its ultrafiltrate in human placental-cord serum. Each curve represents the average colony area for a group of 6 roller-tube cultures.



TEXT-FIGURE 7.—Comparison of horse-serum ultrafiltrate with balanced salt solution in their effect on *14pf* growth. Each curve represents the average colony area for a group of 5 roller-tube cultures, except the HUF+EE curve which represents 4 tubes.

7, however, that chick-embryo extract produced more stimulation in the presence of balanced salt solution than it did when combined with horse-serum ultrafiltrate, indicating that the horse-serum ultrafiltrate suppressed cellular outgrowth. This inhibition is statistically significant, with P less than .05. Thirty percent horse-serum ultrafiltrate alone was in no way superior to balanced salt solution alone.

Thus, the results of these nutritional studies carried out on biological ultrafiltrates showed a growth-promoting effect from chick-embryo

extract ultrafiltrate, but not from horse-serum ultrafiltrate at the concentration tested.

Method Used in Designing Experiments and Analyzing Results [8]

At the outset of an experiment, one needs replicate groups of roller-tube cultures for experimental and control treatments. In the method of replication to be described here, the tubes are represented by their average colony areas. Groups are considered replicates when the following criteria are fulfilled: *a*) Each group contains the same number of tubes, *b*) the total of the tube averages for one group agrees within 10 percent with the total for any other group, and *c*) all the groups have a similar distribution of values. The meaning of these criteria will become clearer when the planning of an experiment is described. An experiment is started when the cultures are 48 hours old, as already described. There are 4 tiny colonies per tube. With the aid of an ocular micrometer, two perpendicular diameters of each colony are measured and recorded in micrometer units. Measurements are easily made because the colonies are small and usually have distinct borders (fig. 2). From these micrometer readings, one may rapidly compute the average colony area in square millimeters for the four colonies in each tube by using an automatic calculating machine. For one tube, this calculation takes about 30 seconds. When the tube averages have been computed, one is ready to arrange them into replicate groups. To illustrate how this is done, we shall use for discussion an experiment on *14pf* normal rat fibroblasts already described (text-fig. 5).

The problem was to compare the growth-promoting effects of whole chick-embryo extract and its ultrafiltrate when mixed with 10 percent horse serum. For this, 3 replicate groups of cultures were needed: one for the extract, one for the ultrafiltrate, and one for the control containing 10 percent horse serum alone. To supply 6 tubes in a group, 18 cultures were needed. The average colony areas in square millimeters for each of these 18 cultures are listed in the first column of table 1. The first step in the classification procedure is to rearrange these tube averages in descending order of magnitude, as in Step 1 of the table. The second step is to subdivide this list of averages into blocks, with the number of tubes in a block equal to the number of treatments to be given. Since 3 treatments are to be given, the list has been divided into blocks containing 3 tubes each, as seen in Step 2. The blocks are numbered with Roman numerals. Each group will receive 1 tube from every block. In order to be impartial about choosing these tubes, numbers 1 through 3 are placed in random order alongside the 3 tube values within each block, as seen in Step 3. The numbers could be randomized mechanically by shaking three numbered objects in a container, but the simplest way is by selection from a table of random numbers, as was done in this case. See statistical tables of Fisher and Yates (*12*, p. 104), and of Cochran and Cox (*11*, p. 422). The fourth and final step is to group together all the values numbered (1) without changing their sequence, all those numbered (2) and numbered (3), forming in this manner 3 separate groups. As seen from the table in Step 4, these 3 groups of tube averages are replicates according to the criteria given. Their totals agree within 1 percent, and they have very similar assortments of values. Note that the top value of each group comes from Block I, the underlying value from Block II, and so on for each value. Thus, in Step 4, the blocks are arranged in horizontal rows, one below the other in the same original order as in Step 3. They are numbered as before with Roman numerals. Since the 3 groups are replicates, it does not matter which is selected for any one treatment. In the experiment under discussion, Group (1) received chick-embryo extract ultrafiltrate, Group (2) served as control, and Group (3) received whole chick-embryo extract.

[8] The material in this section briefly describes the steps performed in arranging *14pf* roller-tube cultures into replicate groups, and the use of an analysis of variance for testing the significance of the final experimental results. The analysis is presented here in condensed, outline form, with elimination of technical terms. To understand the theoretical reasoning behind this procedure, we would recommend the textbooks by Youden (*9*), Snedecor (*10*), and Cochran and Cox (*11*). The purpose of this discussion is to introduce a method which may find wide application in tissue-culture work.

TABLE 1.—*Classification of cultures into replicate groups*

Tube averages	Step 1	Step 2	Step 3
6.36	6. 36	I 6. 36	I 6. 36(1)
6.08	6. 08	I 6. 08	I 6. 08(3)
5.91	6. 05	I 6. 05	I 6. 05(2)
5.88	5. 91	II 5. 91	II 5. 91(3)
5.27	5. 91	II 5. 91	II 5. 91(1)
4.80	5. 88	II 5. 88	II 5. 88(2)
5.44	5. 48	III 5. 48	III 5. 48(1)
4.58	5. 44	III 5. 44	III 5. 44(2)
6.05	5. 27	III 5. 27	III 5. 27(3)
4.28	4. 87	IV 4. 87	IV 4. 87(2)
5.48	4. 80	IV 4. 80	IV 4. 80(3)
4.87	4. 75	IV 4. 75	IV 4. 75(1)
4.73	4. 73	V 4. 73	V 4. 73(2)
4.75	4. 63	V 4. 63	V 4. 63(1)
4.63	4. 63	V 4. 63	V 4. 63(3)
4.58	4. 58	VI 4. 58	VI 4. 58(3)
4.63	4. 58	VI 4. 58	VI 4. 58(2)
5.91	4. 28	VI 4. 28	VI 4. 28(1)

Step 4

	(1)	(2)	(3)
I	6. 36	6. 05	6. 08
II	5. 91	5. 88	5. 91
III	5. 48	5. 44	5. 27
IV	4. 75	4. 87	4. 80
V	4. 63	4. 73	4. 63
VI	4. 28	4. 58	4. 58
Group totals	31. 41	31. 55	31. 27

Three days later, the values within each group are recorded again, and some changes are quite evident, as seen in table 2. For convenience, the control group has been placed first. It will be seen that the values within any one group now vary widely from the values within any other group, and that this variability is clearly reflected among the group totals. In order to decide whether this variability resulted from the treatments given rather than from chance, one compares a value calculated for this variability between groups with a value calculated for chance variability.[6] If the first value is significantly larger than the second, one concludes that the experimental media produced the observed group differences. These variability values may be found by simple substitution in two formulas. Table 3 shows in stepwise fashion how this is done. The final experimental results are recorded at the top of the table, and at the extreme right are the block totals obtained by adding horizontally across each row. Next, is an explanation of the values to be substituted in the formulas, and finally, the two formulas themselves, which may be applied to many types of experiments. (Each formula is a composite of several steps performed in an analysis of variance.)

In order to determine whether the variability between groups (41.92) is sufficiently greater than the chance variability (1.20) to be significant, one finds their ratio: $\frac{41.92}{1.20}=34.93$. The significance of this ratio is found by comparing it with a corre-

[6] By "chance" variability is meant all variability resulting from undetermined causes, such as errors in technique, differences among transplanted fragments, differences among cultures within a block, variations in the physical properties of the roller tubes, and all other unexplained factors leading to variations in growth rate. Its calculated value is the square of the commonly referred-to Standard Deviation.

TABLE 2.—*Final experimental results*

	(2) Control	(1) EUF	(3) EE
I	9. 55	14. 21	17. 00
II	10. 63	11. 50	16. 16
III	8. 73	12. 69	14. 13
IV	8. 01	10. 71	11. 40
V	7. 55	9. 52	13. 93
VI	7. 68	9. 33	11. 25
Group totals	52. 15	67. 96	83. 87

TABLE 3.—*Calculation of variability*

Blocks	Control	EUF	EE	Block totals
I	9. 55	14. 21	17. 00	40. 76
II	10. 63	11. 50	16. 16	38. 29
III	8. 73	12. 69	14. 13	35. 55
IV	8. 01	10. 71	11. 40	30. 12
V	7. 55	9. 52	13. 93	31. 00
VI	7. 68	9. 33	11. 25	28. 26
Group totals	52. 15	67. 96	83. 87	203. 98 (Grand total)

x=individual tube average
T=individual group total
B=individual block total
N=total No. of tubes=18
R=number of treatment groups=3
g=number of tubes in a group=6
P=number of blocks=6
b=number of tubes in a block=3

Σx=the Grand Total=203.98
$(\Sigma x)^2$=41,607.84
Σx^2=sum of squares of each tube average=2,448.52
ΣT^2=sum of squares of each group total=14,372.36
ΣB^2=sum of squares of each block total=7,058.15

$$\text{Variability between groups}=\frac{\frac{\Sigma T^2}{g}-\frac{(\Sigma x)^2}{N}}{R-1}=\frac{83.94}{2}=41.92$$

$$\text{Variability due to chance}=\frac{\Sigma x^2-\frac{\Sigma T^2}{g}-\frac{\Sigma B^2}{b}+\frac{(\Sigma x)^2}{N}}{N-R-P+1}=\frac{11.96}{10}=1.20$$

sponding value in an "*F*" table (table 4). Any single *F* value is specified by the column and row in which it lies. The *F* value corresponding to the ratio 34.93 lies in a vertical column whose numbered heading equals R−1, and in a horizontal row whose numbered heading equals $N-R-P+1$. Therefore, the value found in column 2, row 10 is 7.56. By definition, any experimental ratio larger than this value would occur by chance in only one experiment out of 100. Since the ratio 34.93 is very much greater than the table value, one may be reasonably certain that the observed variability between groups resulted from the experimental treatment given. All of the preceding calculations may be carried out in less than an hour through the use of an automatic calculator.

Although the foregoing calculations show that there was significant growth stimulation in the experiment, it is of particular interest to determine whether the stimulation

TABLE 4.—*1 percent points for the distribution of F**

n_2	n_1 degrees of freedom (for greater mean square)							
	1	2	3	4	5	6	7	8
1	4, 052	4, 999	5, 403	5, 625	5, 764	5, 859	5, 928	5, 981
2	98. 49	99. 00	99. 17	99. 25	99. 30	99. 33	99. 34	99. 36
3	34. 12	30. 82	29. 46	28. 71	28. 24	27. 91	27. 67	27. 49
4	21. 20	18. 00	16. 69	15. 98	15. 52	15. 21	14. 98	14. 80
5	16. 26	13. 27	12. 06	11. 39	10. 97	10. 67	10. 45	10. 27
6	13. 74	10. 92	9. 78	9. 15	8. 75	8. 47	8. 26	8. 10
7	12. 25	9. 55	8. 45	7. 85	7. 46	7. 19	7. 00	6. 84
8	11. 26	8. 65	7. 59	7. 01	6. 63	6. 37	6. 19	6. 03
9	10. 56	8. 02	6. 99	6. 42	6. 06	5. 80	5. 62	5. 47
10	10. 04	7. 56	6. 55	5. 99	5. 64	5. 39	5. 21	5. 06
11	9. 65	7. 20	6. 22	5. 67	5. 32	5. 07	4. 88	4. 74
12	9. 33	6. 93	5. 95	5. 41	5. 06	4. 82	4. 65	4. 50
13	9. 07	6. 70	5. 74	5. 20	4. 86	4. 62	4. 44	4. 30

*Abridged from table 10.7 in "Statistical Methods" (*10*) with permission of the author, G. W. Snedecor, and of his publishers, The Collegiate Press, Inc., Ames, Iowa.

from chick-embryo extract ultrafiltrate (EUF) was significant by itself. Therefore, one subtracts the control-group average from the EUF-group average, and tests the significance of the difference. These averages are calculated from the group totals in table 3. The average for the EUF group is $\frac{67.96}{6}=11.33$, and for the control group $\frac{52.15}{6}=8.69$. The difference $11.33-8.69$ is 2.64. In a manner quite similar to that already described, the observed difference between group averages, 2.64, is compared with that part of the difference which is due to chance. The difference due to chance is obtained by substituting values from table 3 into the formula

$$\sqrt{\text{Chance Variability}\times\frac{2}{g}}, \text{ which equals } \sqrt{1.20\times\frac{2}{6}}=.63.$$

The ratio of the observed difference to the chance difference is $\frac{2.64}{.63}=4.19$. The significance of this ratio is found by comparing it with a corresponding value in a t table (table 5), in much the same way that the F table was used. The t values corresponding to the ratio 4.19 lie opposite the number 2 $(g-1)$, which is 10 in this case, located in the first column labeled "Degrees of Freedom." These t values are 2.228 and 3.169. To find the significance of the experimental ratio 4.19, one selects the largest t value which is exceeded by the experimental ratio. This t value, 3.169, is located in a column headed ".01," which means that the probability of the observed stimulation resulting from chance alone is less than 1 in 100. Thus, there is little doubt that chick-embryo extract ultrafiltrate had a stimulating effect on the *14pf* fibroblasts.[7]

Summary and Conclusions

A simple roller-tube tissue culture method has been described which allows a detailed microscopic study of the nutritional effects of environment on cell colonies grown on glass in the absence of a plasma clot. This method was demonstrated by the use of an easily grown strain of rat fibroblasts, and a simple experimental technique which provides statistically reliable results without the necessity of extreme care in setting up

[7] The authors wish to thank the staff members of the Biostatistics Department of The Johns Hopkins University School of Hygiene and Public Health for their suggestions in the application of statistics to tissue-culture experiments.

TABLE 5.—*Values of t**

Degrees of freedom	Probability of a larger value of t, sign ignored	
	.05	.01
1	12.706	63.657
2	4.303	9.925
3	3.182	5.841
4	2.776	4.604
5	2.571	4.032
6	2.447	3.707
7	2.365	3.449
8	2.306	3.355
9	2.262	3.250
10	2.228	3.169
11	2.201	3.106
12	2.179	3.055
13	2.160	3.012
14	2.145	2.977
15	2.131	2.947

*Abridged from Table IV in "Statistical Methods for Research Workers" (*13*) with permission of the author, R. A. Fisher, and of his publishers, Oliver and Boyd Ltd., Edinburgh.

initial cultures. It is especially well suited to study the early effects of nutritional and growth factors upon cells. Short-term experiments showed that differences in growth rate of these colonies on glass are readily produced by corresponding differences in concentration of beef-embryo extract. Hemoglobin was also found to stimulate growth. The combination of 30 percent horse serum with either 20 percent chick-embryo extract or 20 percent chick-embryo extract ultrafiltrate, inhibited growth relative to the controls containing 30 percent horse serum alone. When all three components were reduced to 10 percent, significant stimulation of areal outgrowth occurred within the first 3 days in the tubes containing chick-embryo extract or its ultrafiltrate. Highly significant stimulation from 20 percent chick-embryo extract or its ultrafiltrate was obtained over a 7-day period without the problem of concurrent inhibition, when these components were combined with 30 percent human placental-cord serum instead of horse serum. When chick-embryo extract at 20 percent was combined with horse-serum ultrafiltrate at 30 percent, marked stimulation of cellular migration and mitoses took place in the outer portions of the colonies with simultaneous loss of cells and hole-formation in the inner portions. In comparison with balanced salt solution, 30 percent horse-serum ultrafiltrate was inhibitory.

The use of colonies rather than fragments to start an experiment, permits one to classify cultures according to area into replicate groups by means of a reliable statistical method. When experimental results are based on area measurements, the group-replication procedure used in setting up the experiment allows these results to be easily and quickly tested for significance. Through the use of a sufficient number of colonies per roller

tube, and roller tubes per treatment group, measuring errors and individual variations between colonies or tubes become relatively unimportant in affecting the experimental results. Although applied to a special case of colonies on glass, the statistical principles of experimental design and analysis described may find usefulness in any work where colony area is the criterion of experimental effect.

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PLATE 118

FIGURE 1.—Portion of a living 18-day-old roller-tube culture of strain *14pf* rat fibroblasts. Note the monocellular membrane extending outward from all sides of the central fragment. × 169

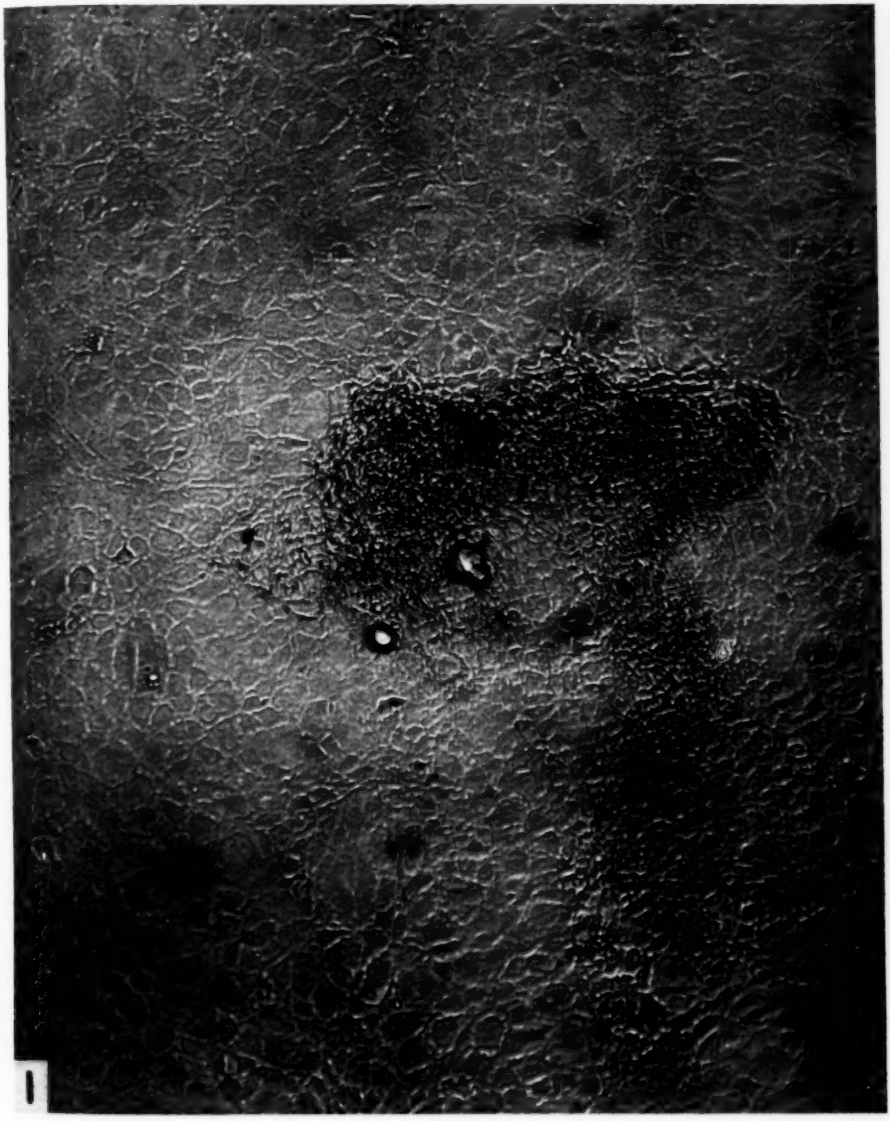

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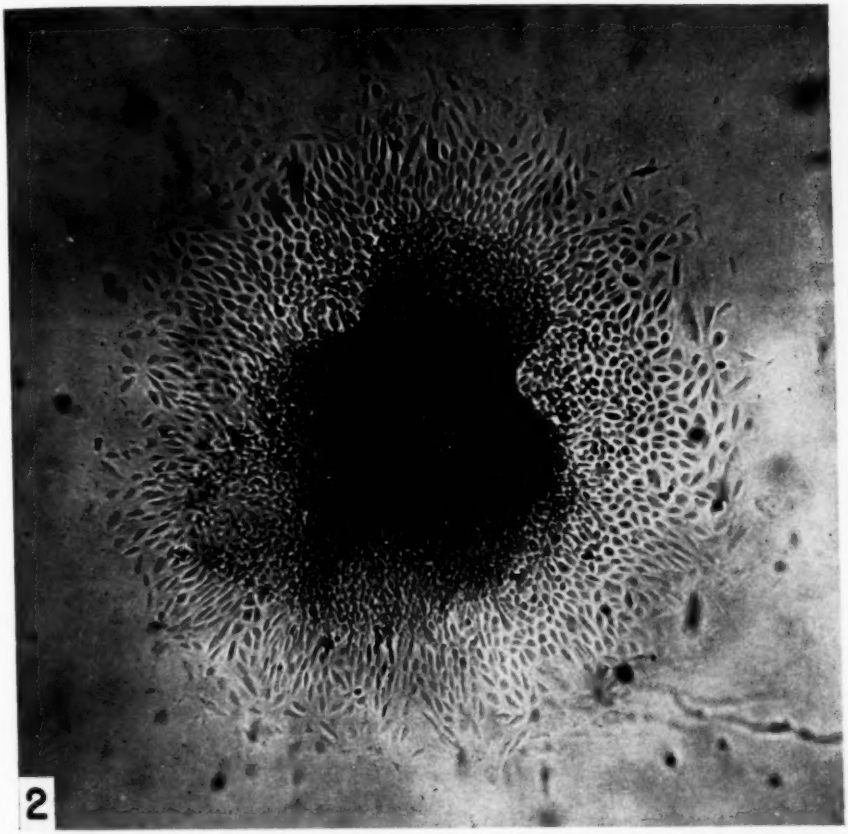
PLATE 119

FIGURE 2.—Living colony from a roller-tube culture of strain *14pf* rat fibroblasts, grown for 48 hours in 10 percent human placental-cord serum. Note the compact, uniform growth. A delicate wavy line from extended membranous processes delimits the colony border in places. The area of the living colony measured 2.4 square millimeters.

FIGURE 3.—Gross appearance of fixed and stained roller-tube cultures of strain *14pf* fibroblasts, showing growth stimulation from one sample of rat hemoglobin. Each pair of tubes consists of the control on the left and the experimental on the right. VandeGrift fixative (*14*); hematoxylin.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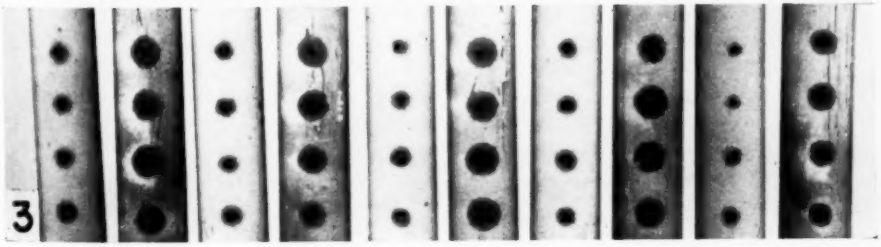
3

Cytological Changes Following Roentgen Irradiation of the Liver in Mice[1,2]

MARGARET E. WILSON[3] *and* ROBERT E. STOWELL,
Department of Pathology and Oncology, University of Kansas Medical School, Kansas City, Kans.

The liver is regarded as a tissue highly resistant to the actions of roentgen rays. Changes in human liver following therapeutic irradiation have been reported (*1*), but often the liver is described as essentially unchanged. Liver damage is described in victims of the atomic bomb explosions (*2*) and has been produced experimentally in rabbits (*3*, *4*). On the other hand, Rhoades (*5*) found that with few questionable exceptions liver was not affected by radiations in her experimental animals. Friedman (*6*) and Ellinger (*7*) have reviewed the major studies of irradiation effects on liver.

The problem of evaluating the effects of roentgen irradiation on the liver is complex because of the sensitivity of the liver to a wide variety of conditions, some of which may occur in irradiated animals. Chief of these conditions is inanition, due either to lowered food intake or to poor utilization of food. The liver responds to inadequate nutritional conditions by a mobilization of fat to the parenchymal cells. Therefore, for example, the investigators (*2*) who studied the atomic bomb victims suggest that the increased fat in the liver may be related to malnutrition, rather than being a direct result of irradiation damage to liver cells. Furthermore, Leblond and Segal (*8*) observed fatty infiltration in livers of irradiated rats whose livers had been shielded from the radiation. The liver may also be influenced by injury to other tissues or alteration of metabolic function. Therefore in the case of total-body irradiation it is difficult to determine whether changes in the liver are due to the irradiation itself, or to damage of other tissues which is reflected in the liver.

The following experiments were designed to avoid, or at least to control, some of these difficulties. The liver was the principal tissue irradiated, and nutritional factors were considered. Furthermore, massive doses of roentgen rays were used in order to obtain some irradiation effects in this resistant tissue.

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[3] *Present address:* Department of Anatomy, New York University College of Medicine, New York, N. Y.

Materials and Methods

Male strain A mice 3 to 4 months of age were used. During irradiation they were tied down on their backs and covered with a lead-rubber shield containing an opening slightly smaller than the liver and of the same general shape. It was possible to check the accuracy of placement of the hole over the liver in animals that were allowed to live several days; any erythematous reaction of the skin was directly over the liver. Furthermore, adjacent tissues (spleen, stomach, intestine, and kidney), occasionally removed for histologic examination, showed no apparent evidence of roentgen-ray damage.

The tissues to be described were irradiated with a 125 kv. therapy machine operating at 10 milliamperes. A 0.5 mm. aluminum filter was used, and the half-value layer value was 1.5 mm. Al. Since the port was small (about 2 cm. in diameter) and the distance from the animal was short (10 cm.), it was possible to give large doses in a reasonably short time. Under the conditions described, the dose in air was about 1,000 r per minute; for convenience, the doses are expressed as the dose obtained at a 1 cm. level within the animal. Livers were exposed to calculated tissue doses of 5,000 r, 7,500 r, 9,000 r, and 12,000 r.

In preliminary experiments with even higher doses from a 225 kv. machine, it was observed that the irradiated animals lost weight, although food was freely available, and although most of the animals did not show many signs of sickness for the first day or so after irradiation. By the third day the weight loss was comparable to that of fully fasted animals; the mean weight loss in both groups was 27 percent of the original body weight. Although the animals irradiated with 12,000 r did not lose as much weight (18 percent by the third day) as the fasted animals, it was a sufficient loss to present a problem as to what would constitute suitable controls. The loss in weight of the irradiated mice might be due to liver damage or to poor absorption of food as much as to lowered food intake; and furthermore, most of the irradiated animals had food in their stomachs at death. Accordingly, it did not seem suitable to employ paired feeding for the controls. Instead, all animals, both control and experimental, were fasted for the duration of each experiment. This was feasible because the experiments lasted for only 2 or 3 days, a period of fast which mice survive. If the experiment lasted for 3 days, all animals were fasted for 3 days. Thus the animals were not all in the same nutritional state when irradiated; one group was irradiated at the time the fast started, another after 1 day of fast, and the third after 2 days of fast. At the beginning of each experiment the mice were isolated in cages with wire-mesh bottoms, and given only water.

The mice were killed at daily intervals for 2 to 3 days after irradiation. The length of time of the experiments was determined by preliminary experiments on the survival of mice after 12,000 r. Ten mice were irradiated as described, and given free access to food. Of these mice, six died during the fourth day after irradiation, and the remaining four

died during the fifth day. Death was probably not due to simple malnutrition, for the weight at death was not as low as that of nonirradiated mice dying of starvation. Two other mice, originally part of this experiment, were not considered in the results because the shield slipped during irradiation; both of them lived several days longer.

A total of more than 60 animals were irradiated, although a few of these could not be included in the final analysis due to a change in position of the shield during the process of irradiation. Tissues were examined from 3 mice for each time-interval after 5,000 r and 7,500 r, and from 7 to 12 mice for each interval after the two higher doses. Since the irradiated animals in any given experiment were fasted for the same length of time, irrespective of the time of irradiation, one group of 3 or 4 fasted controls was adequate for each separate experiment.

Animals were anesthetized with ether and their livers were removed after thorough bleeding. Tissues were fixed in Stieve's fluid (76 cc. of saturated aqueous mercuric chloride, 20 cc. of concentrated formalin, and 4 cc. of glacial acetic acid) for hematoxylin and eosin, Feulgen, and Heidenhain's connective-tissue stains. Frozen sections of other pieces fixed in neutral formalin were stained for fat with Oil Red O.

Preliminary microchemical studies were made of nitrogen, phosphorus, the nucleic acids, alkaline and acid phosphatase, succinic dehydrogenase, esterase, and lipids. With the exception of the lipid study, which substantiated the histochemical observations to be discussed later, the results of the chemical studies were so variable as to be difficult to interpret. Since the data did not contribute to the interpretation of the work discussed in the present paper, they are not included. Preliminary histochemical studies of the phosphatases and esterase also gave variable results.

Results

General.—The animals remained in apparent good health for 1 or 2 days after irradiation. By the third or fourth day many assumed a hunched-up posture and appeared acutely sick; but this also occurred in some of the fasted controls. There was great variation in the responses of individual mice. Some showed few signs of discomfort during the entire experiment. Fed animals often appeared well until just before death, and continued to eat. Diarrhea was observed in some animals.

As previously discussed, most of the experiments employed fasted mice, so that the changes in body weight reflected lack of food. The loss in body weight of fasted, irradiated animals was comparable to that of the fasted controls. However, the liver-weight to body-weight ratio was lower in the irradiated mice. The mean value for the ratio in the control animals was 5.9 percent (range: 4.7 to 7.0). In animals 1 day after irradiation it was 5.1 percent (4.0 to 6.3), and at 2 days it was 4.7 percent (3.9 to 5.4). Since the body-weight changes were comparable in all animals, these data show that the livers of the experimental animals weighed relatively less than those of the fasted controls.

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Cytological measurements.—There was considerable variation in the cellular response of the liver parenchyma in this experiment. Preliminary observations suggested that the cells become smaller after irradiation, although the nuclei and nucleoli are larger. Furthermore, there is appreciably less fat in the cells of fasted, irradiated livers, as compared with the fasted controls. Figures 1 and 2 show the general appearance of control and experimental livers. More precise measurements were made on some of the tissues, to substantiate these observations and to obtain some idea of the extent of the changes. There was little evidence of any difference in tissue response at the different dosage levels employed, with the possible exception of tissue irradiated with 5,000 r. Therefore complete measurements were obtained only for the 12,000 r animals, with fewer measurements at the lower doses for checking purposes. Only the measurements on the tissues receiving 12,000 r will be reported here.

It should be noted that there was no apparent difference in response of various parts of the liver, although there must have been a considerable difference in actual dosages along the axis of the roentgen rays. The fact that the periphery of the liver (which was shielded) reacted in the same way as more central parts may possibly be accounted for by backscatter. Perhaps the lowest dose received by any part of the liver in these experiments was adequate to produce a response to the roentgen rays.

Nuclear and nucleolar volumes were determined on Feulgen-stained sections 4 microns thick. Diameters of at least 100 nuclei were measured for each tissue with a filar-eyepiece micrometer. All of the nucleoli in at least 50 nuclei were measured (always more than 100 nucleoli were measured for each tissue). One nucleus, or the nucleoli in one nucleus, were measured per microscope field. Random sampling was assured by measuring the nucleus that happened to lie nearest to the crossed micrometer threads in each field. Random areas were systematically selected throughout the tissue section, being sure that the tissue section was surveyed two or more times. A few measurements were repeated subsequently, as a check of reproducibility. Volumes were then calculated, assuming that the structures measured were spherical. There are several sources of error in this calculation. Since measurements were made on sectioned tissues, the diameter measured may not always have been the true maximum diameter, especially in the case of the nuclei, whose true diameter is frequently greater than the thickness of the section. This error, however, should be constant in all of the preparations studied. The fact that the nuclei may not all be perfect spheres would also give a constant error. It is felt that these average volumes are a valid basis for comparison of tissues.

The ratio methods of Chalkley (*9*) were used to determine what proportion of the liver cell consisted of nuclear material, of cytoplasm, and of fat. Because of the method of fixation, fat was seen in these preparations as round vacuoles. Since the average nuclear volume is known, from the nuclear measurements, it is possible to calculate approximately the volume of the cytoplasm and of fat in the average cell, and by addition of these

values to determine the volume of the average cell. These calculations are also subject to error. For example, although a great many of the liver cells have more than one nucleus, the value for cell size is calculated on the basis of one nucleus per cell. This was shown to be a constant error. Counts were made of the relative frequency of binucleate cells present in control and experimental tissues, and only rarely was much variation found. An effort was made in a few cases to include the figure for binucleate cells in the calculations of average cell size. The values obtained, although different from the previous figures based on single nuclei, showed essentially the same kind of change in cell size. Since these calculations entailed considerable additional work and gave no additional information, it did not seem worth while to continue the analysis in this manner. Therefore, the figures given for cell volume are based on single nuclei, and are only relative, essentially signifying the amount of cytoplasm and fat per nucleus. Moreover, since the volumes are determined from fixed tissues, they do not represent the size of the living cells.

The cytological measurements on livers of mice irradiated with 12,000 r (table 1) showed considerable variation among animals given the same treatment; however, in most cases the trends were reasonably definite. Both the nuclear and nucleolar volumes increase subsequent to irradiation. In terms of average volume, the nuclear volume is increased about 29 percent and the nucleolar volume about 62 percent by the third day. The Chalkley ratio data show, as would be expected, that the proportion of the liver cell that is nuclear material has increased. This increase reflects both the larger size of the nuclei and the decrease in size of the entire cell. The proportion of the liver cell that is nonlipoidal cytoplasmic material also increases; but volume calculations show that, in absolute terms, the amount of cytoplasm either is not significantly changed or in some cases may be decreased. The proportion of the liver cell that is fat decreases strikingly. From an average of 33.4 percent in the controls it drops to 9.7 percent by the third day. This reflects an absolute decrease in volume of fat, and is the chief reason for the decrease in cell size. It should be remembered that in this experiment the controls are fasted animals. Therefore, compared with normal tissues, the irradiated livers show an increase in fat; this experiment shows rather that following irradiation a fasted animal has less fat in the liver than would be expected. The average nucleo-cytoplasmic ratio (including both cytoplasm and fat) is doubled by the end of 3 days.

Fat changes.—The changes of the fat in irradiated animals are of considerable interest. Not only is there less fat present, as indicated above, but also the distribution of the fat is altered. Figure 3 shows an example of the distribution of fat in the liver of a nonirradiated, fasted mouse. Although there is some variation in the intensity of the reaction, in general all cells contain some stainable lipids. There is even more variation among the irradiated livers. The general tendency is shown strikingly in figure 4; fat is present in cells surrounding the central veins, but lacking

TABLE 1.—*Cytological measurements on liver parenchyma following roentgen irradiation of 12,000 r*

	Nuclear volume μ^3	Nucleolar volume in μ^3		Chalkley ratio data (*percent*)			Calculated volumes in μ^3			N/C ratio
		Individual	Per nucleus	Nucleus	Cytoplasm	Fat	Cytoplasm	Fat	Cell	
Fasted controls.....	187 (167–199)	.24 (.18–.28)	.66 (.57–.76)	6.9 (5.1–9.2)	59.4 (56.0–64.3)	33.4 (29.2–38.7)	1713 (1084–2211)	1027 (559–1769)	2930 (1810–4179)	.07 (.05–.10)
1 day.....	210 (174–235)	.36 (.30–.46)	.98 (.75–1.22)	10.1 (8.0–13.0)	67.4 (58.8–82.5)	22.5 (6.2–32.5)	1402 (1204–1600)	479 (123–766)	2107 (1807–2356)	.12 (.09–.15)
2 days.....	215 (191–237)	.33 (.27–.46)	.79 (.64–.92)	12.1 (10.5–14.0)	70.1 (68.6–71.0)	17.7 (15.0–20.8)	1311 (1105–1555)	265 (91–469)	1791 (1555–2261)	.14 (.12–.16)
3 days.....	241 (215–286)	.39 (.24–.59)	1.07 (.64–1.31)	12.0 (11.3–12.7)	78.2 (75.4–81.8)	9.7 (5.5–12.4)	1567 (1340–1784)	192 (95–272)	2000 (1748–2342)	.14 (.13–.15)

completely from cells at the periphery of the liver lobules. Evidently it is possible that the lipid metabolism of the cells near the portal areas is for some reason more affected by irradiation than those more centrally located.

Cytological changes.—Cytological evidence of degenerative changes is almost always restricted to cells near the portal areas. Sometimes only the nucleus is involved. Figure 5 shows an example of early nuclear damage. In this nucleus the chromatin is organized in thick, densely stained clumps closely applied to the nuclear membrane. More complete nuclear degeneration has also been observed. Figures 6 and 7 give examples of cytoplasmic changes. In figure 6, in which several adjacent cells are affected, the altered cytoplasm shows little change except for some separation from the rest of the cell. In figure 7, however, the altered cytoplasm is darkly stained, and the surface of the nearby nucleus is indented.

More extensive signs of damage are found in animals killed 3 or 4 days after irradiation. A rather frequent observation is the occurrence of hemorrhage in the portal areas (fig. 8). Within the hemorrhagic area can be seen blood cells, degenerating liver-cell nuclei, and cytoplasmic debris.

Restrained controls.—These experiments were all of short duration, animals being killed as early as 18 hours after irradiation. Accordingly it was decided to determine the effects of simply tying down the animal on its back, as was done during the process of irradiation. The mice frequently struggled in this position and it seemed possible that the effects of such stress might influence the liver.

Twelve fasted mice were included in this experiment; six were tied down for 10 minutes 2 days before death, and the remainder were restrained 1 day before death. Tissues from this series were not analyzed in the same cytological detail as the other groups, but unquestionably the livers of tied animals are unlike those of the fasted controls. The most striking difference is the decrease in lipid. Figure 9 shows the liver of an animal fasted for 3 days, and tied on its back for 10 minutes, 18 hours before death. The lipid distribution is patchy, and many cells contain no stainable fat. However, the pattern of distribution is unlike that observed in the irradiated animals. In some livers no pattern was evident; in others, as in the one pictured here, there was no fat in cells surrounding central veins although the cells of the portal areas did contain fat. In other words, the picture is more or less the reverse of the one obtained after irradiation. The results of the various cell measurements show that, in general, the restrained animals are intermediate between the nonirradiated, fasted controls and the irradiated animals. Furthermore, the effects of tying are most apparent within the first day, and by the second day tied animals are more comparable to nonirradiated, fasted controls.

Discussion

In these experiments an effort was made to observe the effects of roentgen irradiation upon the liver without the introduction of other complicating factors. This was difficult to do. Unavoidably, other

tissues than the liver were irradiated. In general, however, important organs such as the spleen and intestinal tract were protected. Possible nutritional effects in the irradiated animals were avoided by the expedient of fasting both experimental and control animals. This was necessary because it would be difficult to determine whether lowered food intake alone produces the poor nutritional status of irradiated animals. Some of the evidence suggested that the simple process of tying the animals down, without subjecting them to irradiation, can produce changes in the liver. Since, however, these changes were transient and quantitatively slight, the fasted irradiated animals were compared with fasted but otherwise untreated controls. With such preliminary experiments it is difficult to interpret the observed changes; in most cases the best that can be done is to describe them as evidence of damage to cells, or cell areas, in animals subjected to roentgen irradiation. Further study will be necessary in order to understand the mechanisms involved in the observed changes.

The results of the cytological measurements show that it is possible to produce changes in the liver by roentgen irradiation of the hepatic region. In almost all cases the greatest relative change occurred by the first day after irradiation, and subsequent days showed a continuation of the already established trends. Since the animals did not die until the fourth or fifth day after irradiation, it is felt that the observed changes are related in some way to the response to irradiation, rather than merely representing premortal changes. Both nuclei and nucleoli increase in size. However, the nucleoli increase relatively more than do the nuclei; therefore, the increase in size of these structures does not appear to be due to simple swelling, as for example from an increased water content. Increase in polyploidy does not appear to be responsible for the nuclear enlargement, for no evidence of mitotic activity was found. If there was an increase in polyploidy it would have to be due to some kind of masked reduplication of chromatin material which could not be observed.

The fat changes are the most striking, both in terms of amount and distribution. It now seems that the previously reported presence of fat in irradiated livers may be explained at least in part by nutritional conditions. The fact that any lipid is present in the irradiated livers is due to fasting. However, the livers of fasted, irradiated mice have less fat than those of untreated fasted animals. For some reason, within one day after roentgen irradiation many of the liver cells lose the fat mobilized to them by fasting. Apparently there is interference with the lipid metabolism and possibly other fundamental functions of these liver cells.

There is further evidence that the cells at the periphery of the lobule are more affected by roentgen rays, for most of the necrosis occurred in cells in this area. This substantiates the observations of Case and Warthin (*1*). These investigators report studies of patients who had been subjected to massive, deep roentgen radiation over the abdominal region. Although these authors concluded that the chief effects of roentgen rays were on the bile ducts, they also observed injury to liver cells at the

periphery of the lobules. On the other hand, Kolodny (*3*), studying rabbits following total-body irradiation, observed numerous scattered necrotic foci. He described hydropic change in the cytoplasm of cells adjacent to the necrotic areas. Tsuzuki (*4*) also studied the rabbit liver after total-body irradiation. He observed that some of the liver cells contained granules and vacuoles, and had nuclei with coarse, granular chromatin. However, only a few of these cells were seen to undergo atrophic degeneration. Liebow, Warren, and DeCoursey (*2*) found only rare examples of necrosis in the victims of the atomic bomb, and this did not follow any consistent pattern. These authors also observed some fatty change, but since it was most common in patients dying after the sixth week the authors felt it was most probably related to malnutrition. Jacquez and Karnofsky (*10*) observed severe liver lesions in young chicks that had died within a few hours of acute large total-body doses of roentgen radiation. They did not find such lesions in chicks dying of delayed irradiation effects.

It is difficult to evaluate these various reports in the literature. In many cases, especially in the older studies, the method of expressing dosage is quite uninformative. Furthermore, the majority of these studies involved total-body irradiation, and most of them did not evaluate the nutritional effects. In the present experiments, it is demonstrated that the liver can be affected by roentgen radiation, although obviously many of the cells are surprisingly resistant, at least so far as visible evidence of change is concerned.

The fact that the pattern of injury is oriented with reference to the lobular pattern of the liver suggests that direct roentgen-ray damage of individual liver cells is not all that is involved. If this were the case it would be expected that degenerative changes would be found at random throughout the lobule. There are a variety of possible explanations for this patterned response. It seems logical to presume that the cells at the periphery of the lobules may be, for some reason, more sensitive. It is known that lowered oxygen tension decreases sensitivity to irradiation; and further it is known that there is an oxygen gradient across the liver lobule, with the highest oxygen tension at the periphery which is closest to the arterial blood supply. These factors may be involved in the apparent greater sensitivity of the peripheral cells. But furthermore, other more generalized reactions may be involved, such as changes influencing the vascular supply and the rate of flow of blood through the lobule, or a change in liver function which would be reflected by further changes in the animals. In addition, the data on the controls which were both fasted and tied down suggest that in short-term experiments of this sort, systemic effects involving such things as the alarm reaction must be considered. From this point of view, it is of interest that Nothacker and Brauer (*11*) found portal hemorrhage in the livers of dogs subjected to continuous infusion of histamine at rates below the shock level. They also described circulatory changes in the livers.

Summary and Conclusions

Doses of roentgen radiation up to 12,000 r (1 cm. depth within the animal) were delivered to the liver region of mice, the rest of the body being shielded. Irradiated animals lost weight at a rate nearly comparable to unfed animals. Therefore, to distinguish between the effects of fasting and irradiation, both experimental and control mice were fasted throughout the experiment. Livers were studied 1, 2, and 3 days after irradiation; at 12,000 r, mice given free access to food died on the fourth and fifth days after irradiation.

Irradiated livers weighed less, in relation to body weight, than control livers. Chalkley ratio measurements showed a decrease in cell size, due principally to a decrease in lipid. Usually both nuclei and nucleoli increased in size, and the nucleo-cytoplasmic ratio was also increased. The fat in the fasted irradiated mouse liver was found in cells at the center of each lobule, while there was little or no fat in the cells near the portal areas. This was in sharp contrast to the more general distribution of fat in the livers of fasted controls. There was further evidence that the cells at the periphery of the lobules are more sensitive to the irradiation effects; isolated necrotic cells and more extensive areas of necrosis and hemorrhage were found most often in the portal areas.

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PLATES

PLATE 120

FIGURE 1.—Liver showing large lipid-filled cells of a control mouse which was killed after fasting 3 days. The fat droplets were dissolved out during the handling of the tissues, but are seen as numerous vacuoles in the cytoplasm. Hematoxylin and eosin. × 350

FIGURE 2.—Liver of a fasted, irradiated mouse with some cells similar to those of figure 1 and many others much smaller containing little, if any, fat. The mouse was killed 2 days after receiving 12,000 r, with a period of fast of 3 days. Hematoxylin and eosin. × 350

FIGURE 3.—Liver of a control mouse fasted for 3 days showing stained fat droplets evenly distributed throughout the tissue. Oil Red O. × 75

FIGURE 4.—Liver of a fasted, irradiated mouse showing distribution of fat principally about central veins. The mouse was killed 2 days after receiving 9,000 r; fasting began at the time of irradiation. Oil Red O. × 75

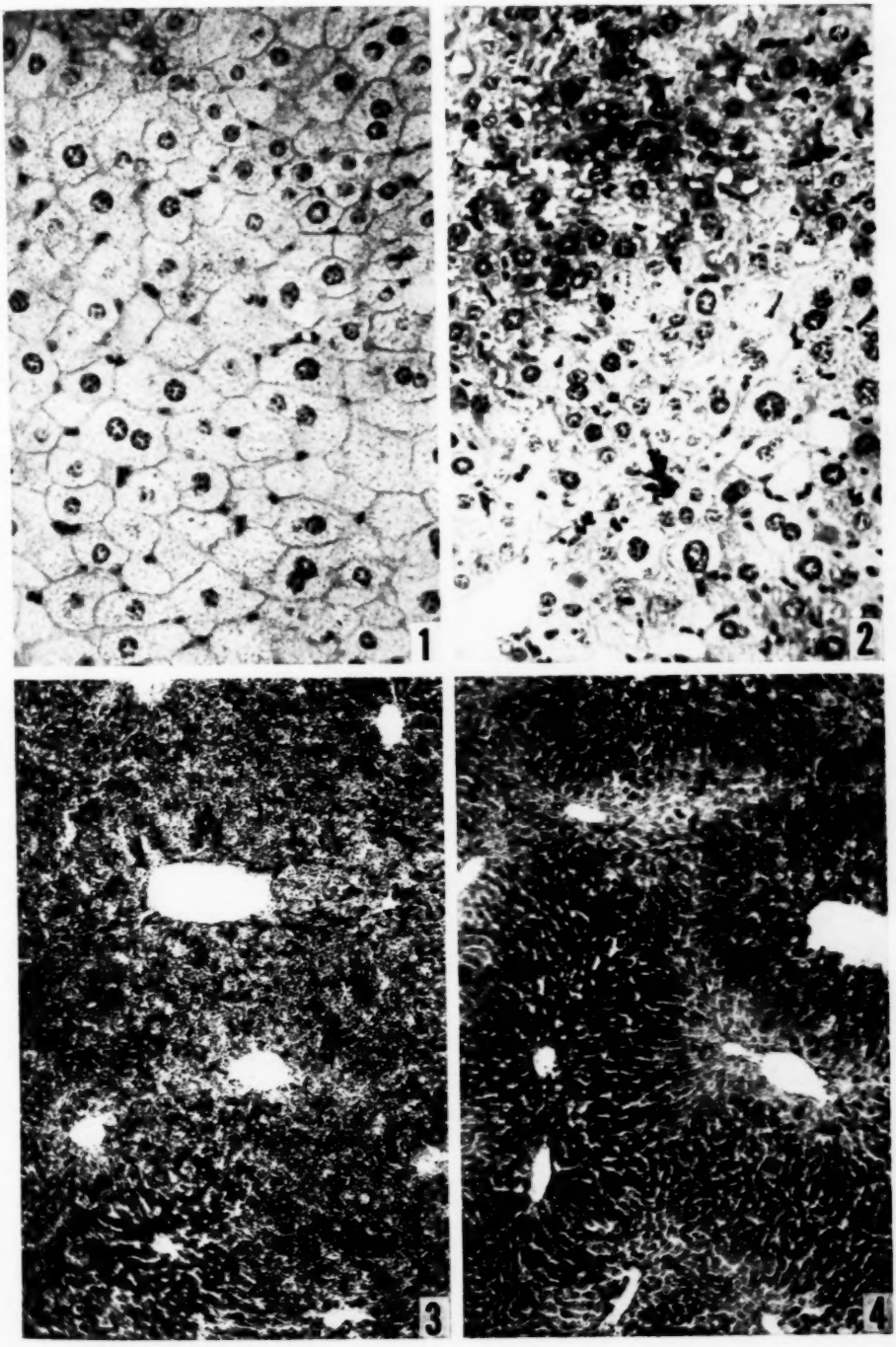
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PLATE 121

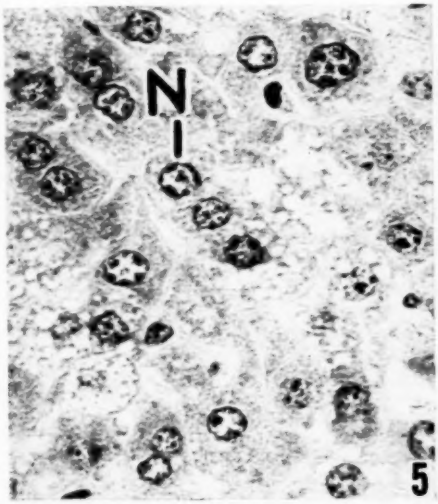
FIGURE 5.—The nucleus of the liver cell at the center of the figure (N) is atypical; the chromatin is in heavily stained clumps applied to the nuclear membrane. The mouse was killed 3 days after receiving 12,000 r; fasting began at the time of irradiation. Hematoxylin and eosin. × 700

FIGURE 6.—A cytoplasmic change is seen in three of the cells above the center of the field with peculiar cytoplasmic areas (C). Although these areas appear normal, each is clearly set off from the rest of the cytoplasm. The mouse was killed 3 days after receiving 12,000 r; fasting began at the time of irradiation. Hematoxylin and eosin. × 700

FIGURE 7.—The large cell below the bile duct at the left contains two changed regions in its cytoplasm (C). Both are intensely stained, and one indents the surface of the nearby nucleus. This mouse was killed 1 day after receiving 9,000 r; the animal was fasted for 2 days. Heidenhain's azocarmine connective-tissue stain. × 700

FIGURE 8.—To the right of the bile duct is an area of portal hemorrhage; a blood vessel above the bile duct opens into this area. Included in the area are many darkly stained red blood cells and degenerating liver-cell nuclei. The granules of the background are predominantly cytoplasmic debris. This mouse was killed 3 days after receiving 12,000 r; fasting began at the time of irradiation. Heidenhain's azocarmine connective-tissue stain. × 350

FIGURE 9.—This liver is from a mouse that was fasted 3 days, and tied down on its back for 10 minutes 1 day before death. There is less fat present than in the non-irradiated, fasted control (fig. 3). Moreover, unlike the irradiated tissues (fig. 4) the distribution of fat is patchy; the only groups of cells without stainable fat are those around central veins. Oil Red O. × 75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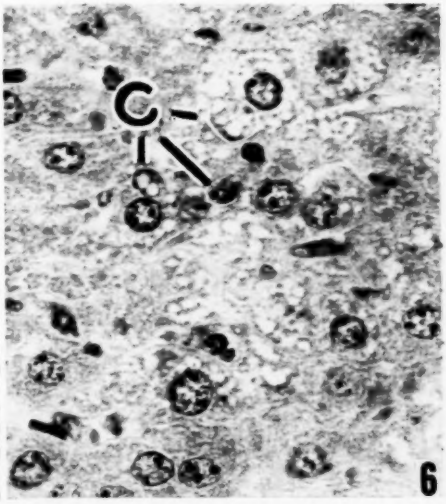
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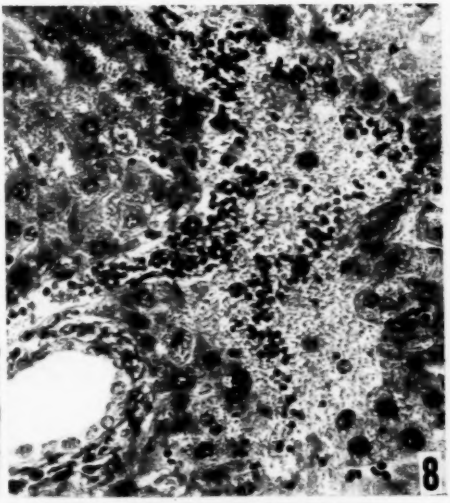
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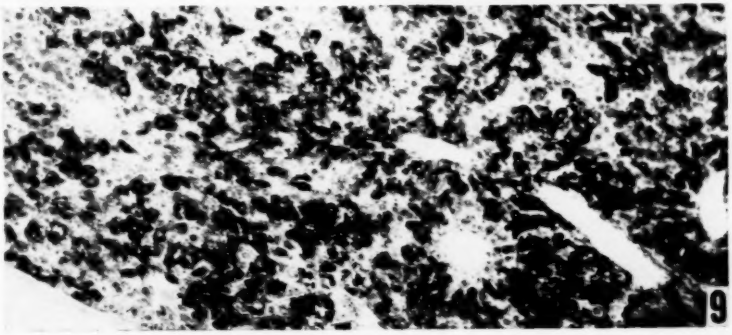
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Tumors of the Liver and Urinary Bladder of the Dog After Ingestion of 2-Acetylaminofluorene[1]

HAROLD P. MORRIS *and* WILLARD H. EYESTONE,
National Cancer Institute,[2] *Bethesda, Md.*

Wilson, DeEds, and Cox (*1*) were the first to demonstrate the carcinogenicity of 2-acetylaminofluorene (AAF) in rats in chronic toxicity studies. They found tumors in the urinary bladder, renal pelvis, liver, pancreas, lung, subcutaneous tissue, colon, and breast within 3 to 11 months following commencement of the administration of the compound. Since this initial work, many other investigators have reported the carcinogenicity of this compound in rats. Tumors have been observed in several other species, including rabbits [Bonser and Green (*2*)], mice [Armstrong and Bonser (*3*), Foulds (*4*), and Kirby (*5*)], cats [Green and Bielschowsky (*6*) and Skoryna *et al.* (*7*)], fowls [Bielschowsky and Green (*8*) and Peacock and Peacock (*9*)], and dogs [Allison *et al.* (*10*)].

The object of this experiment was 1) to ascertain the susceptibility of the dog to the induction of tumors by AAF; 2) to make available large quantities of excretory materials from which to study the metabolism of AAF; and 3) to provide material from another species with which to compare the metabolic pathway of AAF with that of the rat.

Experimental Procedure

Five dogs were used in the experiment: Foxy, Brownie, Shorty, Copper, and Midas. Three of the dogs (Foxy, Brownie, and Shorty) were small short-haired animals, resembling terriers, and each was about 12 months of age when placed on the diet. The other two dogs (Copper and Midas) were beagle litter mates, born in the laboratory. They were placed on the diet at 6 months of age.

The animals were housed individually in metabolism-type cages that contained partial wooden floors for their comfort. The dogs were fed a diet, prepared in the laboratory, that contained the following (by weight): Commercial casein 330, cerelose (commercial dextrose) 330, lard 210, cod-liver oil 10, corn oil 10, dehydrated brewer's yeast 60, wheat-germ meal 10, $Ca_3(PO_4)_2$ Technical 32.5, and salt mixture 185$_a$ (*11*) 7.5. Six hundred mg. of AAF was added per kg. of this diet from the beginning of the experiment in July 1944 until August 1947. The amount of AAF

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[2] National Institutes of Health, Public Health Service, Federal Security Agency.

was increased to 1.2 gm. per kg. of diet at that time and maintained at the higher level until the end of the experiment. The AAF was first dissolved in warm lard and this solution was thoroughly incorporated in the diet mixture with an electric dough mixer.

The weights of the animals, taken at monthly intervals, varied from approximately 5 to 20 kg.; therefore, it was deemed desirable to express the AAF per unit of body weight so that the intake of the various dogs could be studied on a comparable basis. Feedings were daily, except Sunday—a double portion being fed on Saturday. Records of total weekly intake were kept. The amount of food offered the animals was restricted, when necessary, to prevent them from becoming overweight. On the other hand, there were times when individual animals refused to eat the carcinogenic diet and it was necessary to revert to the basal diet to allow them to regain their health. This was especially true of the dog Brownie, who consequently consumed the smallest total amount of the carcinogen during the experimental period.

Euthanasia was accomplished with a lethal dose of nembutal for each of the five dogs prior to autopsy.

Experimental Results

The average daily intake of AAF by dog Brownie (table 1) ranged from 1.00 to 9.06 mg. per kg. of body weight during seven periods. These values represent the daily intake of AAF during the entire period. This dog ingested the noncarcinogenic diet for about half of the entire experimental period (column C, table 1). The average daily intake of AAF per kg. of body weight usually ranged between 1.0 and 3.7 mg. This dog consumed all of the carcinogenic diet for the first 5 months of the experiment. During that period the intake per kg. of body weight was higher than for any of the later periods. This dog failed to develop tumors either in the liver or in the urinary bladder.

Dogs Midas and Copper (table 1) were litter mates. They consumed the largest amounts of AAF amounting to almost 200 grams each for the 45 to 46 months during which AAF was offered them. The AAF-containing diet was not refused during the experiment, but the dogs were taken off the AAF during the last 7 to 8 months. Surgical biopsies several months prior to necropsy had definitely shown extremely nodular livers in both animals. It was thought that these nodular livers would develop hepatomas without additional AAF. This proved to be the case as described below. Hence, the animals were taken off the AAF-containing diet for several months prior to autopsy.

Midas and Copper were larger than the other three animals in this experiment, yet their average daily intake of AAF per kg. of body weight was only slightly, if any, greater than that of dogs Shorty and Foxy.

All four dogs developing tumors of the liver and urinary bladder consumed much larger amounts of AAF than Brownie, who did not develop any tumors.

TABLE 1.—*Average daily intake of 2-acetylaminofluorene (AAF) for each of 5 dogs calculated for periods of approximately 300 days in length*

Dog	A	B	C	D	E	F‡	G§
	Total* period	On AAF	Off AAF	Mean weight of dog	Total† AAF intake	AAF intake/ kg. dog	Avg. daily AAF intake/ kg. dog
	(*days*)	(*days*)	(*days*)	(*kg.*)	(*gm.*)	(*gm.*)	(*mg.*)
Brownie (male).....	161	161	0	8.4	12.25	1.46	9.06
	293	140	153	9.3	6.44	0.69	2.93
Started 7/24/44	270	133	137	8.9	8.96	1.01	3.74
Autopsy 10/9/51	258	48	210	8.4	2.16	0.26	1.00
	294	188	106	8.6	7.04	0.82	2.79
	297	119	178	8.1	6.51	0.80	2.69
	53	35	18	8.0	1.57	0.20	3.77
Shorty (female)....	160	160	0	10.1	11.88	1.18	7.38
	301	301	0	8.9	23.58	2.65	8.80
Started 7/24/44	294	294	0	8.2	30.03	3.66	12.45
Autopsy 3/29/50	280	280	0	7.9	31.36	3.97	14.18
	90	90	0	7.7	8.29	1.08	12.00
Foxy (female).....	160	160	0	6.2	7.30	1.18	7.38
	294	294	0	5.9	18.91	3.21	10.92
Started 7/24/44	285	266	19	6.3	21.42	3.40	11.93
Autopsy 1/31/52	303	112	191	6.0	6.76	1.13	3.73
	281	175	106	6.1	10.84	1.78	6.33
	301	301	0	6.0	20.56	3.43	11.40
	166	70	96	5.8	3.97	0.68	4.10
Midas (male).....	294	294	0	13.8	39.45	2.86	9.73
	301	301	0	16.1	34.24	2.13	7.08
Started 1/1/45	294	294	0	14.0	42.96	3.07	10.44
Autopsy 3/2/51	294	294	0	14.2	44.10	3.11	10.58
	296	217	79	14.7	31.63	2.15	7.26
	131	0	131	14.0	0	0	0
Copper (male).....	294	294	0	14.1	41.06	2.91	9.90
	301	301	0	14.2	40.57	2.86	9.50
Started 1/1/45	301	301	0	12.7	45.13	3.55	11.79
Autopsy 3/31/52	259	259	0	12.5	38.85	3.11	12.01
	296	217	79	11.8	32.55	2.76	9.32
	160	0	160	12.0	0	0	0

*Between the first and second periods Brownie, Shorty, and Foxy went 30 months, and Copper and Midas 20 months without AAF. In the entire experiment records are incomplete for the following numbers of days: 89 for Brownie, 30 for Shorty, 39 for Foxy, 19 for Midas, 48 for Copper. These are not included in the total.

† During the first period (161 days for Brownie, 160 days for Shorty and Foxy, 294 days for Midas and Copper) the dogs received AAF at a level of 0.6 mg./gm. diet. Thereafter all dogs received the drug at a level of 1.2 mg./gm. diet.

‡ $F=\frac{E}{D}$

§ $G=\frac{F}{A}$

A summary of the AAF ingestion by the five dogs is presented in table 2. Two male and two female dogs developed tumors. One male (Brownie) did not develop tumors. The average AAF intake, calculated only for periods of AAF ingestion for Brownie, was approximately one-half as great as for the other four dogs. This one factor seems to be one of the most important differences between the dogs developing tumors and the one that did not develop tumors. The length of the observation period

TABLE 2.—*Summary of experiments on 2-acetylaminofluorene (AAF) ingestion in the group of dogs that developed tumors*

	Sex	Duration of experiment*	Period		Weight range	Total intake AAF	Average daily intake AAF/kg. of body weight†
			On AAF	Off AAF			
		(*months*)	(*months*)	(*months*)	(*kg.*)	(*gm.*)	(*mg.*)
Brownie	M	87. 2	27. 5	26. 7	5. 5–13. 7	45	3. 2
Shorty	F	68. 5	37. 5	0. 0	7. 5–10. 9	105	10. 9
Foxy	F	91. 0	45. 9	13. 7	4. 8– 6. 8	90	8. 3
Midas	M	74. 3	46. 7	7. 0	13. 0–19. 3	192	8. 3
Copper	M	75. 3	45. 7	8. 0	11. 4–16. 5	198	9. 5

*Total time elapsed from beginning of experiment to autopsy.
†Total AAF intake per total no. days per mean weight of dog.

was almost as great for Brownie as for Shorty, and considerably longer than that of the other three dogs.

The average daily food intake per kg. of body weight for dog Shorty was 7.38 to 14.18 mg., with the daily intake per kg. over 10 mg. for most of the experiment (table 1). This dog ate the diet containing AAF throughout the experiment and consumed a total of 105 gm. of AAF during an experimental period of 68.5 months. Tumors of the liver and urinary bladder developed in this dog after a somewhat shorter period of feeding than for any of the other animals.

Dog Foxy (table 2) was on experiment for a total of 91 months. This period was divided into 45.9 months on AAF diet and 13.7 months off AAF diet, plus the 30 months when no AAF was offered the animals. The average daily intake of AAF per kg. of body weight was from 3.7 to 11.9 mg. The daily intake was approximately 11 mg. for three periods, and about half that amount for four periods. The total intake of AAF was approximately 90 gm. during the experimental period. This dog consumed carcinogen approximately three-fourths of the experimental period. Tumors of both the urinary bladder and the liver were present at autopsy at the end of the experiment.

TABLE 3.—*Summary of experiments on the ingestion of 2-acetylaminofluorene (AAF) by a group of beagle dogs not developing tumors*

	Duration* of experiment	Average mean weight of dog	Total AAF intake	Average daily AAF intake per kg. of body weight
	(*months*)	(*kg.*)	(*gm.*)	(*mg.*)
Dog 1	27. 3	6. 7	36. 41	7. 3
Dog 2	27. 0	5. 6	36. 94	8. 9
Dog 3	27. 2	8. 1	37. 62	6. 5
Dog 4	27. 3	6. 3	32. 24	7. 1

*These dogs were given AAF during the entire experiment.

A summary, in table 3, is given of experiments on four other beagle dogs, observed for 27 months, in which death occurred from distemper and in which no tumors developed. The average daily AAF intake per kg. of body weight was as high for two dogs as for dogs in the other experiment in which tumors did develop. These four dogs satisfactorily consumed the AAF-containing diet throughout the 27 months of the experiment. It was unfortunate that these four animals could not have been kept until they also developed tumors. No tumors of the liver, or of the urinary bladder, have occurred in six other dogs fed this basal food for 5 to 5½ years.

Pathology

A complete pathological report is given for the dog named Copper, followed by descriptions of the remaining four animals only as essential differences were seen. The post-mortem examinations included the central nervous system. Tissues were fixed in both Zenker formol fluid and in 10 percent formalin.

Liver

Gross description.—The organ was extremely enlarged and weighed 1,179 grams, more than three times the average normal liver weight for a dog of its body weight. The entire surface was nodular, some of the nodules measuring four to five centimeters in diameter and extending considerably above the surface. The larger nodules were red in color and contained considerable blood, or were grayish yellow and friable. Smaller nodules, located deep in the parenchyma, were lighter yellowish to grayish in color. The liver cut with resistance. The larger, more friable masses could be enucleated from the surrounding liver. Occasionally, there was a confluence of several nodules. No direct extension of the tumors to other viscera was seen, although there were fibrous adhesions to the adjacent diaphragm, omentum, and wall of the stomach. The tumors were scattered in a rather haphazard manner, but were most prevalent at the periphery of the lobes. The common bile duct was patent, and approximately 20 cc. of dark-green viscous bile was present in the gall bladder. The mucosa of the gall bladder contained numerous mucous cysts.

Microscopic description.—Sections were taken from sixteen different areas of the liver. Eight of these areas proved to be hepatocellular carcinoma, while the remaining eight showed varying degrees of hyperplasia with mild cirrhosis, marked fatty metamorphosis, and focal areas of necrosis. In the hyperplastic areas, the liver cells were enlarged and the nuclear chromatin was prominent. At the periphery of the hyperplastic nodules there was a condensation of cells, seemingly caused by the pressure of the expanding growth. Occasional strands of fibrous connective tissue separated tumor nodules from the adjacent liver tissue. Large areas of fatty change were seen in the noncancerous areas of the liver. The fat in these cells usually occurred in large single globules, completely obscuring the cytoplasm.

The cellular and architectural patterns of the tumor nodules were variable. Some closely resembled well-differentiated liver parenchymal cells, arranged in cords, but without lobular orientation or bile ducts. There were single or multiple rows or cords of cells, covered at their periphery with a thin flattened endothelium forming a sinusoidal pattern with the adjacent cords of cells (fig. 2). The tumor cells were slightly larger than normal liver cells; there was margination of nuclear chromatin and usually one prominent nucleolus (fig. 3). Binucleate cells were seen with relative frequency. At the periphery of this type of nodule there was a condensation and atrophy of the adjacent liver cells and a few thin strands of connective tissue cells. Occasionally entrapped bile ducts were seen in the condensed liver tissue near the periphery of the nodule.

Another type of nodule stood out more in contrast to the surrounding liver tissue because of its deep basophilic staining. The tumor cells were smaller, had very little cytoplasm and were oval to polygonal in shape (fig. 4). The nuclei were dense, sometimes indented, and contained one large nucleolus. Mitotic figures were numerous. Around blood vessels, the cells had a tendency to line up radially in relation to the vessel wall. In general, however, the architectural pattern for these areas consisted of cells arranged in a cordlike fashion separated by spaces lined with endothelium.

A third type of tumor nodule was composed of cuboidal cells having a centrally placed nucleus, arranged in small ringlets of 10 to 20 cells, covered with flattened endothelium (fig. 5).

Intrahepatic metastases appeared to have played a part in the extension of the tumors since large tumor emboli were frequently seen in blood vessels (fig. 9).

Fatty changes were noted in nearly all areas of the tumor. In contrast to the noncancerous tissue, the cytoplasmic vacuoles of the cells in cancerous areas were smaller and much more numerous.

Urinary Bladder

Gross description.—Numerous papillomas were present, being larger and more extensive posteriorly in the region of the trigonum vesicae. There were fewer papillomas toward the anterior surface of the bladder wall. The larger papillomas (fig. 11) were eroded and revealed hemorrhages on their surface. These growths ranged in size from small grapeseed lesions to mushroom-type forms with a prominent stalk. The largest of the papillomas measured about 2 cm. in diameter. The orifices of the ureters were patent, and no evidence of tumor tissue was seen in the ureters.

Microscopic description.—There was extensive hyperplasia of the mucosa in all sections observed. In areas adjacent to the papillomatous lesions, metaplastic changes to the squamous type of epithelium were noted (fig. 13). The central stalk of connective tissue, with its blood vessels, supporting the papillomas and its arborizations was derived from the submucosa (fig. 12). The papillomas were covered by many

layers of transitional-type epithelium and on the surface, metaplasia to squamous-like cells had occurred (fig. 14). Intercellular bridges could not be demonstrated between these cells. In the depth of some of these epithelial proliferations, infoldings had caused them to appear as glands on cross section (fig. 15). The cytoplasm of the squamous-like cells, particularly near the surface, or the central areas in the case of infoldings, had periodic acid-Schiff-positive material in them that was glycogen. The periodic acid-Schiff-positive material was not present in the diastase-treated control sections fixed in Zenker's formol fluid.

At the base of one of the papillomas, there was a localized invasion of tumor cells into the submucosa and muscular layers (fig. 17). No metastases from the lesions of the urinary bladder were noted.

Lung

Gross description.—There were several subpleural grayish-pink nodules, ranging in size from 5 to 10 mm. in diameter. The parenchyma of the lungs was otherwise normal in appearance.

Microscopic description.—Four sections of the subpleural nodules seen in the gross examination revealed metastatic hepatocellular carcinoma. At the periphery of the smaller nodules, the outline of the walls of blood vessels could be seen, indicating hematogenous spread. Large nodules apparently caused compression of the surrounding lung parenchyma. The enlarged nuclei and nucleoli of the tumor cells were especially prominent in these metastatic lesions (fig. 10). Cytoplasmic vacuoles were present in many of the cells, indicating the probable presence of fat. The PAS reaction did not reveal the presence of glycogen in the tumor cells. Focal areas of necrosis, usually near the center of the tumor mass, were seen as in the primary tumors.

Spleen

Gross description.—The spleen appeared slightly enlarged and bled freely from the bulged surface of the cross section. Five raised nodules, ranging in size from 5 to 15 mm., were located just beneath the capsule. These were reddish pink in color and resembled infarcts.

Microscopic description.—The lymphoid follicles were very active. There was extensive subintimal calcification of the larger arteries. Many histiocytes laden with iron pigment were seen in the sinusoids. The subcapsular nodules noted in the gross proved to be infarcted areas. No evidence of metastatic tumor was found.

Stomach

Gross description.—No gross lesions were noted.

Microscopic description.—The submucosa of the cardiac portion of the stomach was infiltrated with lymphocytes which in focal areas formed a follicular architecture.

Intestine

Gross description.—There was a deep-brownish discoloration of the small intestine extending from the pylorus to the ileum. The intensity of this discoloration diminished toward the ileocecal junction. On cross section of the intestine it was apparent in the muscle coats. There was very little material in the lumen, but a small amount of brownish-tinged mucus was present throughout. The mucosa of the large intestine was thrown into folds and the formed ridges showed hemorrhagic inflammation. Blood was present in the lumen. The intestine appeared to be thickened throughout its length.

Microscopic description.—Sections taken from the first portion of the duodenum showed marked granular degeneration of the circular and longitudinal muscle fibers. Numerous histiocytes containing brownish-yellow granules were seen intermingled among the degenerating muscle fibers (fig. 21). The changes seen gave the impression that the cytoplasmic fibers had contracted and broken up into fine granular debris. These granules reacted negatively to the periodic acid-Schiff reaction. Sections taken from the jejunum showed much less severe changes, and in the large intestine they were not seen at all. The large intestine showed dilation of the mucosal capillaries, and a few small hemorrhages.

Comparison of "Copper" With Other Dogs in Experiment

Liver

Gross comparisons.—The liver of dogs Midas, Shorty and Foxy all contained tumors. The liver of dog Midas was as extensively involved as that of dog Copper (fig. 1). On the surface of one of the larger nodules, the liver capsule was torn and a large clot had formed over it. There was considerable blood in the abdominal cavity. The hemorrhage appeared to have resulted from the rupture of the liver capsule covering the tumor nodule. In dog Foxy, only the left lobes of the liver were extensively involved with tumor. There was one massive tumor measuring $12 \times 7 \times 5\frac{1}{2}$ cm. in the left lateral lobe. The right lateral lobe was slightly enlarged and contained scattered nodules up to 5 mm. in diameter. The liver of dog Shorty was similar to that of Foxy, but was less involved with tumor. It contained several nodules up to 3 cm. in diameter and one large mass measuring 6×3 cm. The liver of dog Brownie did not contain tumors. It was approximately normal in size, was deep yellow in color, and contained a few small, dark-red foci in the parenchyma.

Microscopic comparisons.—Microscopic examination of liver tumors from dogs Midas and Foxy revealed close similarity to those from dog Copper. Those from dog Shorty showed variation in cellular and architectural pattern and extensive cirrhosis (fig. 6). The tumor cells located in areas of most marked cirrhosis were pleomorphic, consisting chiefly of giant cells with up to 10 nuclei, and bizarre arrangements of nuclear chromatin (fig. 7). Unusual filiform arrangements of simulated liver cords

were seen in other hepatomatous areas (fig. 8). In addition to these variations in tumor pattern, sections from several other tumors in the liver of dog Shorty were quite similar to those seen in dog Copper. The liver of dog Brownie revealed extensive fatty metamorphosis.

Urinary Bladder

Gross comparison.—Dogs Midas, Foxy, and Shorty all had numerous papillomas on the urinary-bladder mucosa, which were similar to those described for dog Copper; however, none were seen in dog Brownie. In the latter animal, the bladder wall was thickened and the mucosa was dark red in color. The prostate gland was enlarged. The prostatic portion of the urethra was inflamed.

Microscopic comparison.—Lesions in the urinary bladder of dogs Midas, Foxy, and Shorty were similar to those described for Copper, with the exception that no invasion of the bladder wall by the tumor cells was seen. Sections of the bladder wall from dog Brownie revealed marked lymphoid infiltration into the submucosa and cystitis cystica (fig. 16). The cystic mucosa was also seen in several sections of ureter that were examined. The prostate gland showed diffuse chronic inflammation with numerous small abscesses. The mucosa of the prostatic urethra showed cystic changes similar to those seen in the urinary bladder.[3]

Lung

Metastatic tumors were not seen in any of the remaining animals of the group.

Spleen

Similar in most respects to dog Copper, with the exception that no subintimal calcium deposition was seen in the arterial walls in dog Brownie.

Ovary

Two small tubular adenomas, located under the ovarian capsule, were seen in dog Foxy (fig. 20).

Gastrointestinal Tract

Similar in most respects to dog Copper.

Pancreas

Gross comparison.—The pancreas of dog Foxy contained numerous miliary grayish-colored nodules ranging up to 4 mm. in diameter. These nodules were present throughout the length of the gland. No similar lesions were seen in the other dogs of the group.

[3] In addition to the changes described in the lower urinary tract, this animal had chronic pyelonephritis. The lesions found in the urinary system of this animal are frequently seen in older dogs, and since they were found in only one of five treated animals, would not seem likely to have resulted directly from the carcinogenic diet.

Microscopic comparison.—The nodules described above had the microscopic appearance of adenomas of acinar cells. Some of these contained basophilic cells that were smaller than normal acinar cells and stained lighter, although their appearance and architectural arrangement resembled acini (figs. 18 and 19). Occasional mitotic figures were seen in these areas. Other nodules were composed of large cells having abundant eosinophilic granular cytoplasm and resembling acinar cells that had retained their secretion granules. In both types of nodules, there was compression of the adjacent parenchyma of the pancreas.

The changes in pancreas of dog Foxy were not seen in any of the other dogs in the experiment.

Discussion

An average daily intake of 1.6 mg. of 2-acetylaminofluorene (AAF) per kg. of body weight of a rat for a period of 18 weeks has been found in this laboratory to result in a low incidence of approximately 5 percent of tumors. The dog Brownie ingested daily on the average twice that amount of AAF for a period of 27.5 months and failed to develop tumors after an observation period of 87 months. The four dogs that developed tumors in this experiment ingested on the average 5 to 6 times the amount of AAF ingested by the rats in the above-mentioned experiment. It would appear that for the dog the level of ingestion of the AAF in these experiments compared with that of the rat was well above a minimum level for tumor-production in the rat. The average daily intake of 0.4 mg. of AAF per kg. of body weight of the rat was found to be noncarcinogenic. The dog may have greater ability to inactivate the carcinogen, or the tissues of the dog may possess a greater resistance to the induction of tumors by AAF than do those of the rat.

These experiments demonstrate the long period required in the dog for the induction of tumors by AAF. This might be considered as approximately half the normal lifetime of this species of animal, and is much longer than the approximately 2¼ years that Hueper *et al.* (*12*) found for the induction of tumors of the urinary bladder in dogs fed β-naphthylamine, a compound whose chemical structure closely resembles AAF.

Allison *et al.* (*10*) induced tumors in the liver of dogs in a period of only 6 months. There is no question that nodular livers were found by these investigators. There is a question, however, as to whether the lesions were hepatomas, and because of their rapid development there would appear to be other factors involved. This is especially noteworthy inasmuch as we have been unable to produce similar lesions in a second group of five beagle dogs after 27 months of continuous ingestion of AAF at a level of 0.12 percent of the diet.

The dogs of either sex were susceptible to the development of cancer in at least two distant sites after the ingestion of AAF. The metabolites present in the excretory products formed in the dog following the ingestion of AAF [Weisburger *et al.* (*13*)] are now being investigated. So far, two metabolites have been isolated and identified as 2-acetylamino-7-hydroxyfluorene and 2-aminofluorene. Both were present in the urine to the

extent of only 1 or 2 percent of the amount ingested. These findings suggest that the dog may not only have the power to deacetylate AAF, but may also oxidize some AAF by the same pathway as does the rat, as shown by Bielschowsky (*14*).

The isolation of such small amounts of two AAF metabolites from the urine of the dog leads to speculation in regard to the real probability that other major metabolic pathways for AAF exist, some of the products of which may possess greater carcinogenic activity. Extensive studies to discover such other metabolic processes which may exist are now in active progress in this laboratory. Allison and Wase (*15*) report that excess riboflavin produced increased excretion of unidentified conjugates of 2-aminofluorene from the urine of the dog as well as nonvolatile phenols that they believe to be detoxified products of aminofluorene ingestion.

The presence of a microscopic encapsulated granulosa-cell tumor in the ovary and pancreatic adenomas in dog Foxy is of interest since a variety of tumors are induced in rats following the ingestion or painting of this compound (*1*, *16*). These tumors cannot be definitely said to arise as a result of the effects of the ingestion of AAF, although it might well have been the causative agent. Bonser and Green (*2*) noted an adenocarcinoma of the ovary in one of eight rabbits treated with 2-acetylaminofluorene, and they could not absolutely rule out AAF as the inducing agent.

Summary and Conclusions

Epithelial tumors of the liver and urinary bladder were induced in dogs following the oral administration of 2-acetylaminofluorene. The experiment included five dogs, four of which developed the tumors in both locations from 68 to 91 months after the beginning of the experiment. Animals developing tumors received a total of from 90 to 198 grams of the carcinogen. The one animal not developing tumors ingested a total of 45 grams of AAF. The extent of tumor formation was directly related to the amount of carcinogen consumed, being most marked in those animals that received a total of nearly 200 grams during the feeding period. Another group of four animals receiving a total of 32 to 37 gm. during a 2¼ year period did not develop tumors.

Carcinoma of hepatic parenchymal cells, which metastasized to the lung, was produced. Varying histologic patterns were seen in the tumors of the liver. No tumors of bile-duct origin were seen. Multiple papillomas were produced in the urinary bladder, and in one animal, invasion of the submucosa and muscle by the tumor cells was seen. The ureter and renal pelvis were not involved in the hyperplastic and neoplastic processes.

Granular degeneration of smooth-muscle cells occurred in the wall of the small intestine and was most prominent in the duodenum near the ampulla of Vater, and became much less apparent in the jejunal region. Follicular gastritis was present in all animals. Subintimal calcification of the splenic artery and its main tributaries was associated with chronic passive congestion of the spleen.

Adenomatous hyperplasia of pancreatic acinar cells was noted in one animal. A microscopic encapsulated granulosa-cell tumor of the ovary was observed in one dog.

It is apparent that a large amount of 2-acetylaminofluorene is necessary, when fed orally, to induce tumors in dogs, and that there is a long latent period. The induction of tumors in dogs by the use of this carcinogen provides an opportunity to obtain large quantities of excretory materials to facilitate the isolation and study of metabolic products of 2-acetylaminofluorene and to facilitate comparison of the metabolism of this compound in the rat and dog.

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PLATES

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PLATE 122

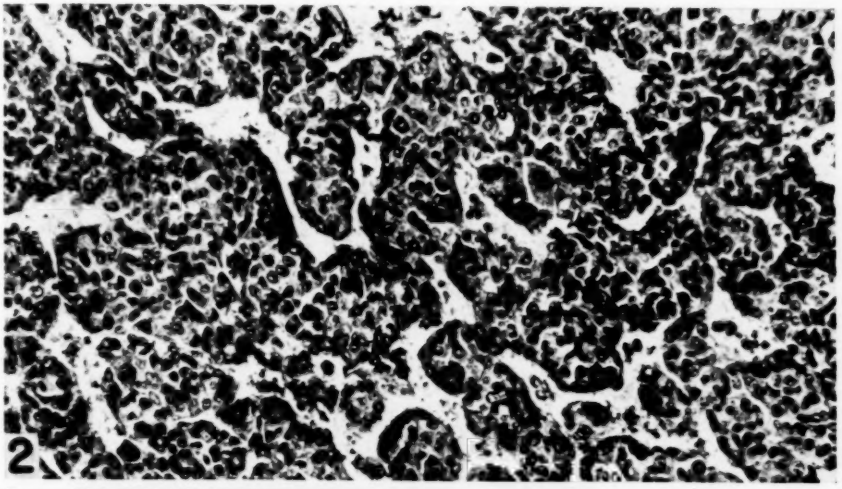
FIGURE 1.—Ventral view of the liver from dog Midas showing almost complete obliteration of the organ by tumor.

PLATE 123

FIGURE 2.—A section of hepatocellular carcinoma showing cords of cells lined up in single or multiple rows. Hematoxylin and eosin. × 230

FIGURE 3.—Hepatocellular carcinoma. High-power view of figure 2. Note enlarged nucleoli and margination of nuclear chromatin. Hematoxylin and eosin. × 600

FIGURE 4.—Hepatocellular carcinoma showing less tendency to liver architecture and composed of smaller cells with very little cytoplasm. Mitotic figures were numerous in this type of tumor nodule. Hematoxylin and eosin. × 230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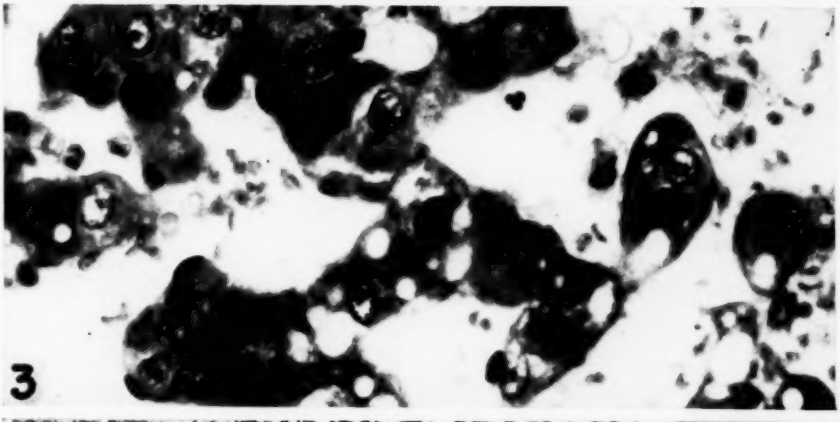
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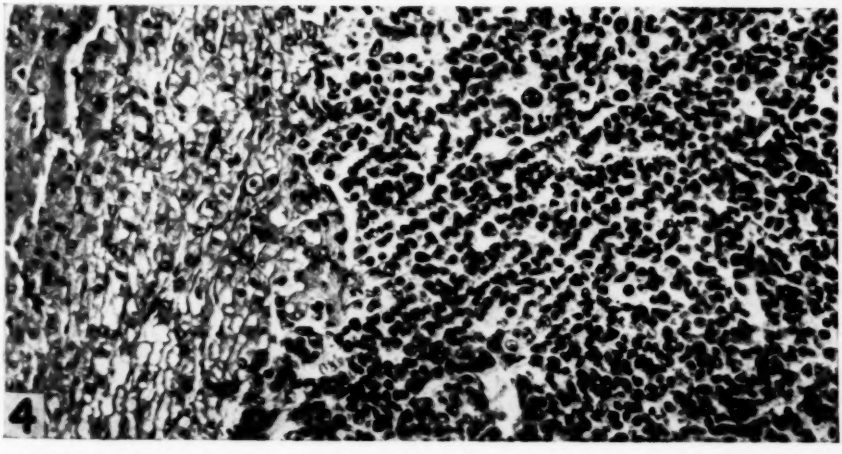
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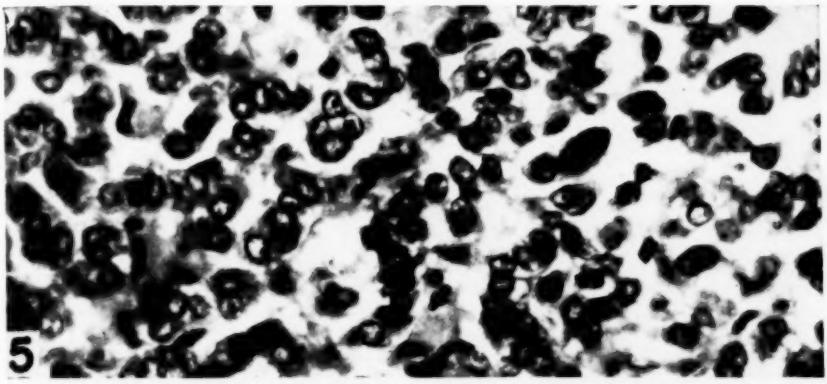
PLATE 124

FIGURE 5.—Hepatocellular carcinoma. Cords of cells show a tendency to form ringlets. Hematoxylin and eosin. × 600

FIGURE 6.—Liver section from dog Shorty showing cirrhosis with inclusion of bile ducts. Hematoxylin and eosin. × 230

FIGURE 7.—Hepatocellular carcinoma from dog Shorty showing marked pleomorphism of cells. Hematoxylin and eosin. × 270

FIGURE 8.—Hepatocellular carcinoma from dog Shorty showing papillary type of of architecture. Hematoxylin and eosin. × 145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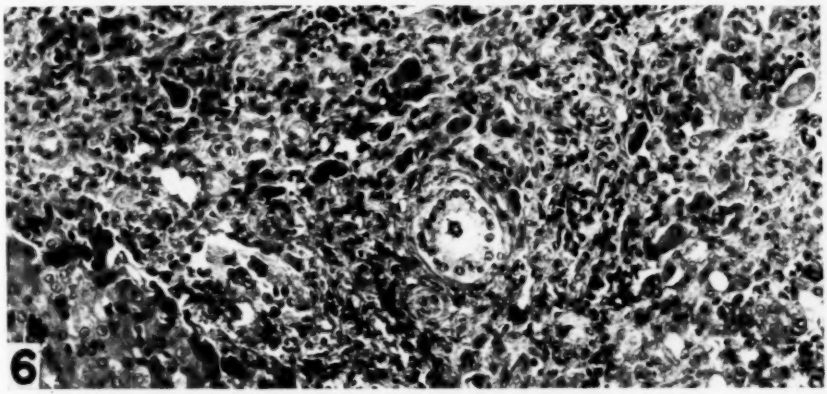
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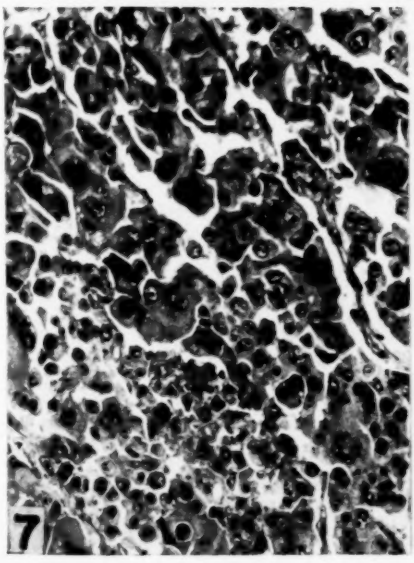
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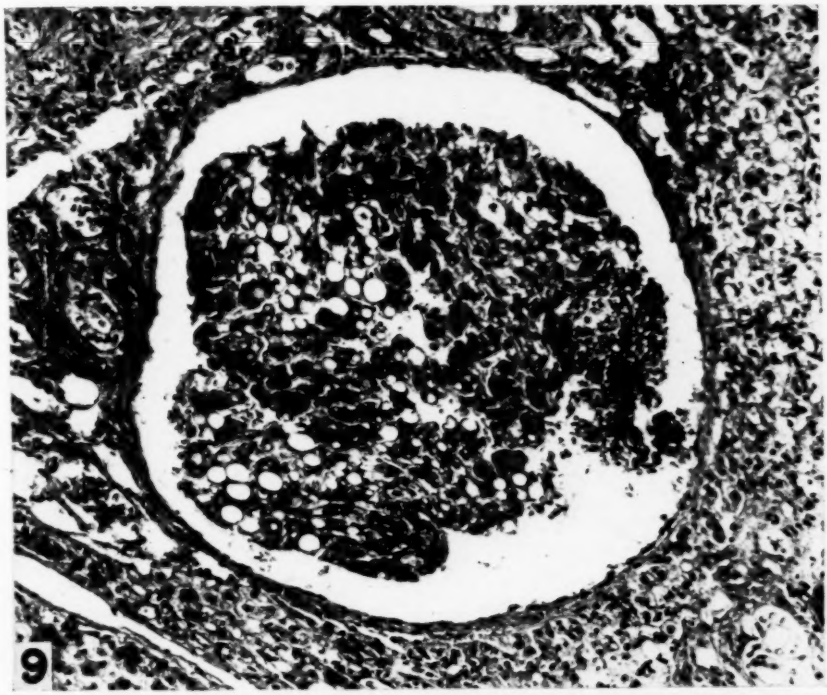
PLATE 125

FIGURE 9.—A large tumor embolus in a hepatic vein. Dog Copper. Hematoxylin and eosin. × 175

FIGURE 10.—Hepatocellular carcinoma, lung metastasis. Dog Copper. Hematoxylin and eosin. × 230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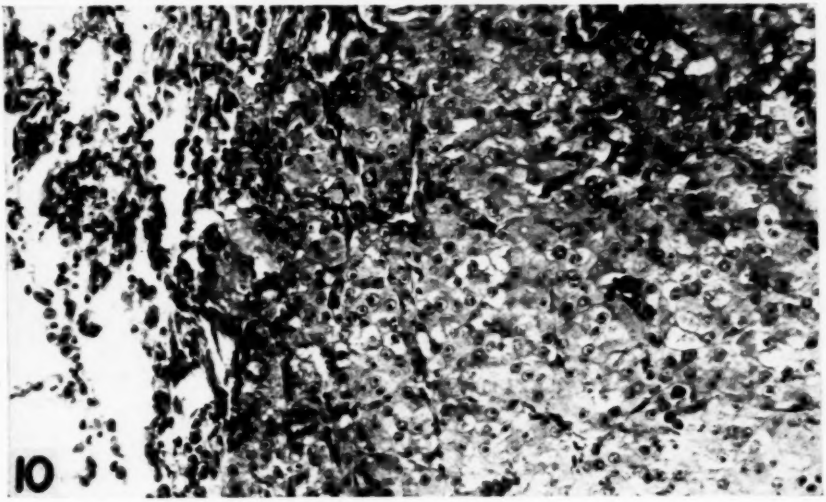
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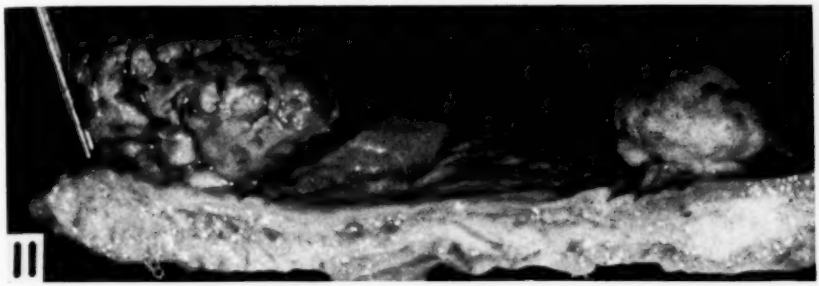
PLATE 126

FIGURE 11.—Papillomas arising from the epithelium of the urinary bladder. Dog Shorty. × 1½

FIGURE 12.—Papilloma of the urinary bladder. Dog Copper. Hematoxylin and eosin. × 12

FIGURE 13.—Epithelium of urinary bladder near the base of a papilloma, showing hyperplasia and metaplasia to squamous type. Dog Copper. Hematoxylin and eosin. × 60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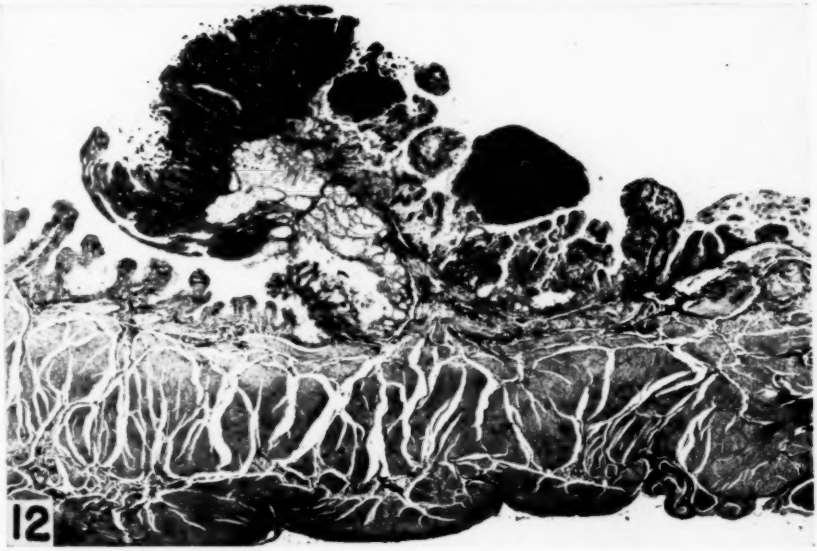
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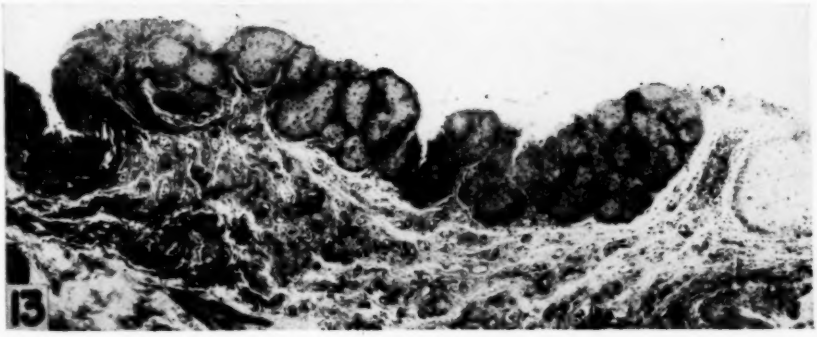
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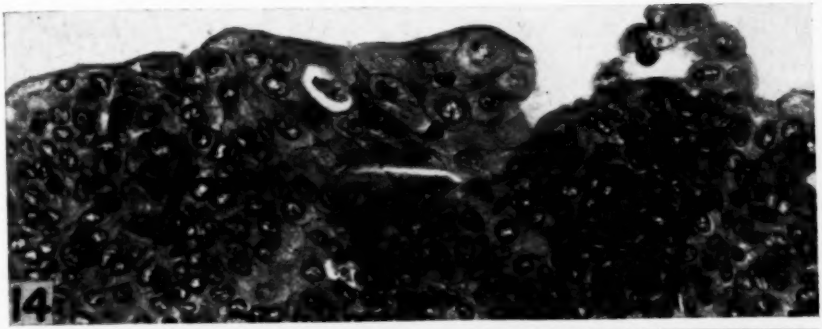
PLATE 127

FIGURE 14.—Surface of a papilloma of the urinary-bladder epithelium showing transition to squamous type. Dog Copper. Hematoxylin and eosin. × 500

FIGURE 15.—Pseudoglandular structures in papilloma of the urinary bladder showing more advanced state in which surface cells degenerate. Dog Copper. Hematoxylin and eosin. × 500

FIGURE 16.—Epithelium of the urinary bladder of dog Brownie showing intraepithelial cysts and chronic inflammation of the submucosa. Hematoxylin and eosin. × 23

FIGURE 17.—Urinary bladder. Dog Copper. Local invasion of tumor cells at the base of a papilloma. Hematoxylin and esoin. × 500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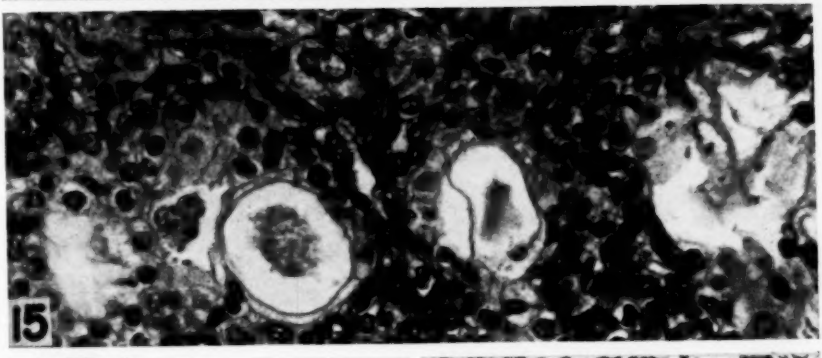
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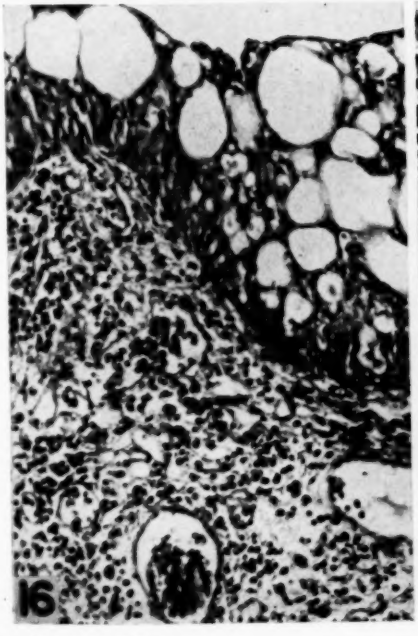
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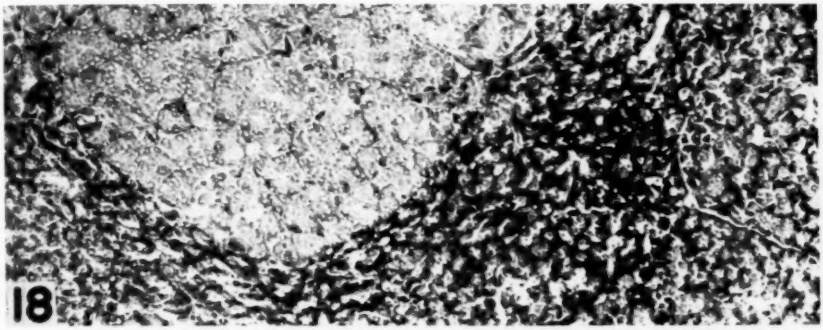
PLATE 128

FIGURE 18.—Adenoma of pancreatic acinar cells. Dog Foxy. Hematoxylin and eosin. × 120

FIGURE 19.—Adenoma of pancreatic acinar cells. High-power view of figure 18. Note mitotic figures. Hematoxylin and eosin. × 380

FIGURE 20.—Ovarian tubular adenoma. Dog Foxy. Hematoxylin and eosin. × 380

FIGURE 21.—Granular degeneration of smooth muscle fibers in small intestine. Note histiocytes laden with debris. Hematoxylin and eosin. × 720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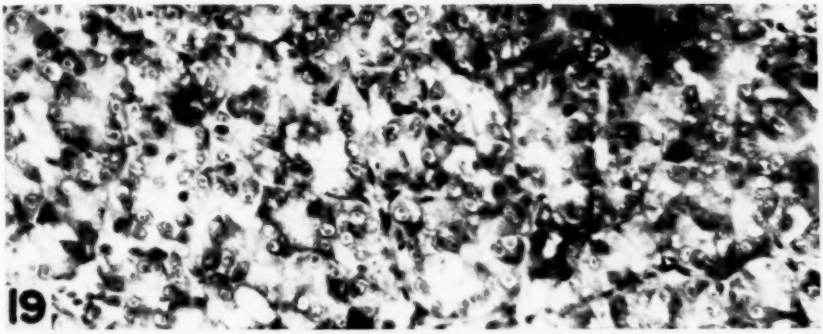
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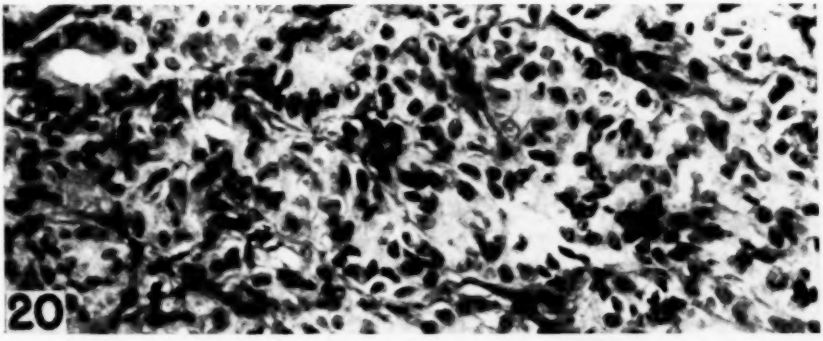
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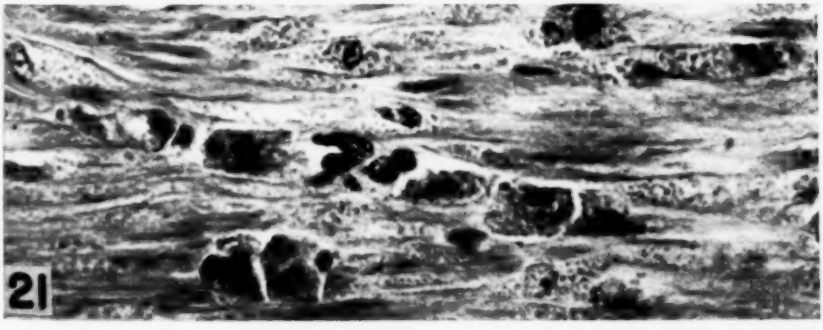
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Dose-Response Relations in Experimental Transmission of Avian Erythromyeloblastic Leukosis. II. Host-Response to Whole Blood and to Washed Primitive Cells[1,2]

EDWARD A. ECKERT,[3] DOROTHY BEARD, *and* J. W. BEARD, *Department of Surgery, Duke University School of Medicine, Durham, N. C.*

With an Appendix on Histopathology by B. R. BURMESTER, *Regional Poultry Research Laboratory, East Lansing, Mich.*

Erythromyeloblastic leukosis (*1*) is a highly malignant neoplastic disease of chickens caused by a filtrable virus and manifested in the occurrence of large numbers of primitive cells in the circulation. The disease is readily transmissible (*2*, *3*), under proper conditions, by means of the virus which is present in high concentrations (*4*) in the plasma after filtration. In a recent report (*5*), there were described the quantitative aspects of the dose-response relations in experimental transmission with the agent in filtered plasma. Whole blood, which contains not only the virus but the primitive cells as well, is likewise effective (*6*), as would be expected, in transmitting erythromyeloblastic leukosis. While there have been observed certain apparent differences between the transmission potentialities of the virus in filtered plasma and those of inoculums containing the primitive cells, no quantitative studies have been made either to define or to measure these differences. Moreover, there has appeared to be but small expressed appreciation not only of the profound differences between transmission by virus infection and the transplantation of a malignant tumor, but of the significance of host-response with respect to the occurrence of a virus disease in chickens and the reaction of the chicken population to transplantable cancer. In order to complement the quantitative studies on virus transmission and to establish the character and extent of possible differences between virus and cell potentialities, an

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investigation has been made of host-response to graded doses of whole blood and of washed primitive cells from donors with erythromyeloblastic leukosis. The results of this work are described in the present report.

Materials and Methods

The disease investigated here was the same as that which has been under study in this laboratory as described in previous reports (*4*, *5*). The origin of the strain of virus (*7*) and the lineage of the passages through the 25th consecutive experiment have also been given in an earlier paper (*5*). In a more recent report (*8*) the lineage has been shown through the 37th experiment, and the continuation leading to the present experiments is shown in Chart 1 of this report. The pathology of the disease, particularly with respect to the characters of the primitive cells, has been described by Dr. B. R. Burmester in an appendix to the present paper.

The procedures and materials used in the work described here were identical with those employed in the studies on titration of the virus (*5*) with the few exceptions that pertain to special handling of the blood and cells.

A group of five individual studies were made, of such magnitude as to give promise of results of satisfactory accuracy. Whole blood was used in two experiments and washed cells in two others; in one case, experiment 51, the response to whole blood was compared with that to washed cells from the same blood. The results of a series of six earlier experiments made on a smaller scale are grouped in text-figure 3 for consideration along with the principal data. Interrelations of these small experiments, the numbers of which are given with respect to the various passages of the disease, will be found in Chart 1 of (*5*). The procedures given here pertain to the five large experiments, and significant deviations occurring in the small studies are given in a later paragraph.

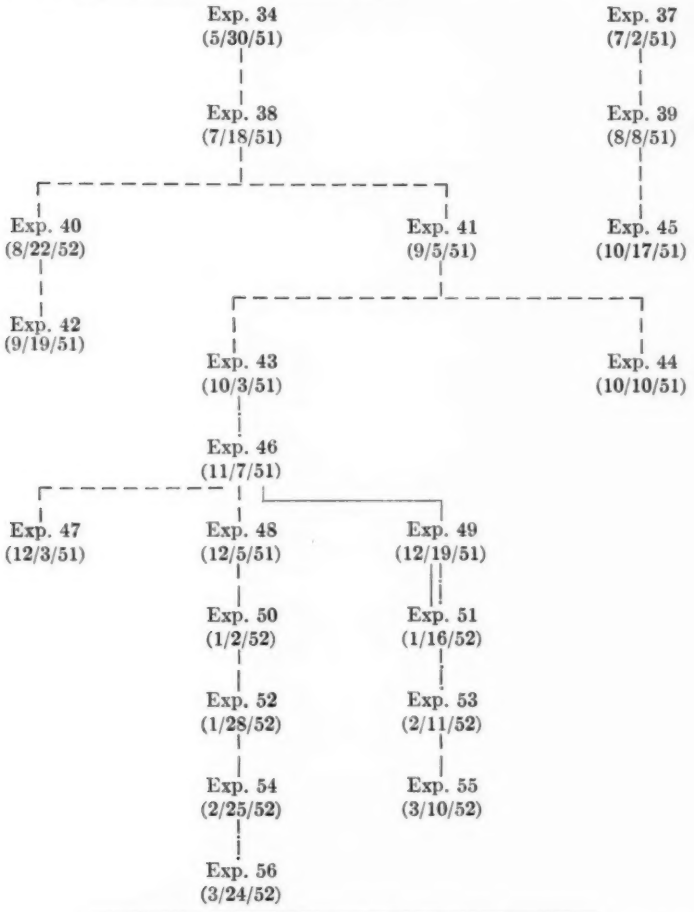
For each experiment, a pool of blood was drawn by heart puncture from several chickens, with the use of heparin (*5*) as the anticoagulant, and passed through several layers of surgical gauze for the removal of an occasional small clot. The blood was chilled in a container immersed in ice as soon as compatible with other procedures. The number of donors yielding the pools in the various experiments were 3, 4, 5, 6, and 4 in experiments 13, 49, 51, 53, and 56, respectively.

In order to obtain washed cells, the chilled blood was centrifuged in 50-ml. glass tubes in the cold at 600 $\times$ g for 20 minutes. After the plasma was removed by pipetting, the cells were resuspended in 9 volumes of BSA-Simms' solution (*5*) and sedimented again in 250-ml. glass bottles at 700 $\times$ g for 10 minutes. This washing process in the 250-ml. bottles was carried out for a total of six times. The final dilution factor, therefore, was about 10^8 with respect to the cell volume. The washed cells were then diluted with BSA-Simms' solution to the original volumes of the blood. Cell counts of the whole blood and of the suspensions of cells gave values differing by five percent or less.

For preparation of the inoculums, serial dilutions, as indicated in table 1, were made of the whole blood or washed cells with BSA-Simms' solution. Every effort was made to keep the preparations cold by immersion of containing vessels in ice water. Bleeding was carried out at about 7 a. m. in the morning of the experiment, and all procedures, including inoculation, were concluded by 12:30 p. m. Cell counts were made in Neubauer counting chambers after dilution in calibrated (Bureau of Standards) red-blood-cell pipettes with phloxin as previously described (*5*, *9*). Inoculations were made intravenously in 0.1 ml. volumes in 3-day-old New Hampshire chicks, except in experiment 13 in which the inoculums were 0.05 ml. The procedures for making smears, diagnosis, and other routine studies were the same as those already described (*5*, *10*). The numbers of test hosts are shown in table 1.

The small experiments, 2 to 7, all were concerned with the study of whole blood and differed from the larger ones principally in the fluids used for making the dilutions for

Chart 1. Origin and interrelations of material used in the various experiments. The unbroken lines (——) represent passage by whole blood; the broken dash lines (— —) were passages by means of cell-free plasma; and the broken dash-dot lines (—·—) were passages by washed primitive cells.



inoculation. For experiments 2 and 3, Tyrode's solution [4] was prepared at *p*H 7.4 to 7.8 on the day before the experiment, sterilized with ultraviolet light (*10*), and placed in the refrigerator overnight. It was learned, after experiment 3 was completed, that the *p*H may rise, as in this instance, to 8.6. Simms' solution (*11*), kept in rubber-stoppered flasks, was employed subsequently in the preparation of other saline diluents after sterilization with ultraviolet light. Dilutions were made in experiments 4 and 5 with a mixture of 1 volume of chicken serum and 3 volumes of Simms' solution. The serum was obtained from adult hens that were apparently normal. BSA-Simms' solution (*5*) was used in experiments 6 and 7 as in the larger experiments described above. All diluents contained 1 ml. of heparin per 100 ml. Inoculations were made intravenously in 0.1 ml. volumes in 3-day-old New Hampshire chicks. The doses

[4] NaCl, 8.0 gm; KCl, 0.20 gm; $CaCl_2$, 0.2 gm; $MgCl_2.6H_2O$, 0.10 gm; $NaH_2PO_4.H_2O$, 0.05 gm; $NaHCO_3$, 1.00 gm; glucose, 1.00 gm; and distilled water to make 1,000 ml.

TABLE 1.—*The results of experiments 13, 49, 51, 53, and 56*

Experiment No.	Inoculum		Dose log ml. whole blood or cell suspensions														
			−1	−1.3	−2	−2.3	−3	−3.3	−4	−4.3	−5	−5.3	−6	−6.3	−7	−8	−9
13	Whole blood	No. chicks inoculated		125		125		124		126		125		125			
		Corrected total inoculated		124		124. 6		123. 9		125. 9		125		125			
		No. positive inoculations		103		87		73		76		51		44			
		Percent positive inoculations		83. 06		69. 82		58. 92		60. 37		40. 80		35. 20			
49	Whole blood	No. chicks inoculated	90		90		90		90		90		90		90	90	
		Corrected total inoculated	87		88		89		89		88		88		89	87	
		No. positive inoculations	85		70		60		48		44		38		31	9	
		Percent positive inoculations	97. 70		79. 55		67. 42		53. 93		50. 00		43. 18		34. 83	10. 34	
53	Washed cells	No. chicks inoculated	80				80				80		120		120	120	120
		Corrected total inoculated	79				78				75		118		118	118	119
		No. positive inoculations	43				45				25		35		20	5	2
		Percent positive inoculations	54. 43				57. 69				33. 33		29. 66		16. 95	4. 24	1. 68

56	Washed cells	No. chicks inoculated	80				80				80		120		120	120	120
		Corrected total inoculated	80				80				79		120		120	109	119
		No. positive inoculations	47				45				27		35		27	24	16
		Percent positive inoculations	58. 75				56. 25				34. 18		29. 17		22. 50	22. 02	13. 45
51	Whole blood	No. chicks inoculated	90				90				90						
		Corrected total inoculated	87				90				88						
		No. positive inoculations	62				54				25						
		Percent positive inoculations	71. 26				60. 00				28. 41						
51	Washed cells	No. chicks inoculated	90		90		90		90		90						
		Corrected total inoculated	88		88		89		86		87						
		No. positive inoculations	66		56		45		36		25						
		Percent positive inoculations	75. 00		63. 64		50. 56		41. 86		28. 74						

were 10^{-1} through 10^{-6} ml. whole blood in all cases, except experiment 7 in which the range 10^{-1} through 10^{-9} was employed. Each dilution of each material was inoculated into 20 chicks. The numbers of donors in experiments 2 to 7 were 3, 2, 4, 4, 11, and 3, respectively.

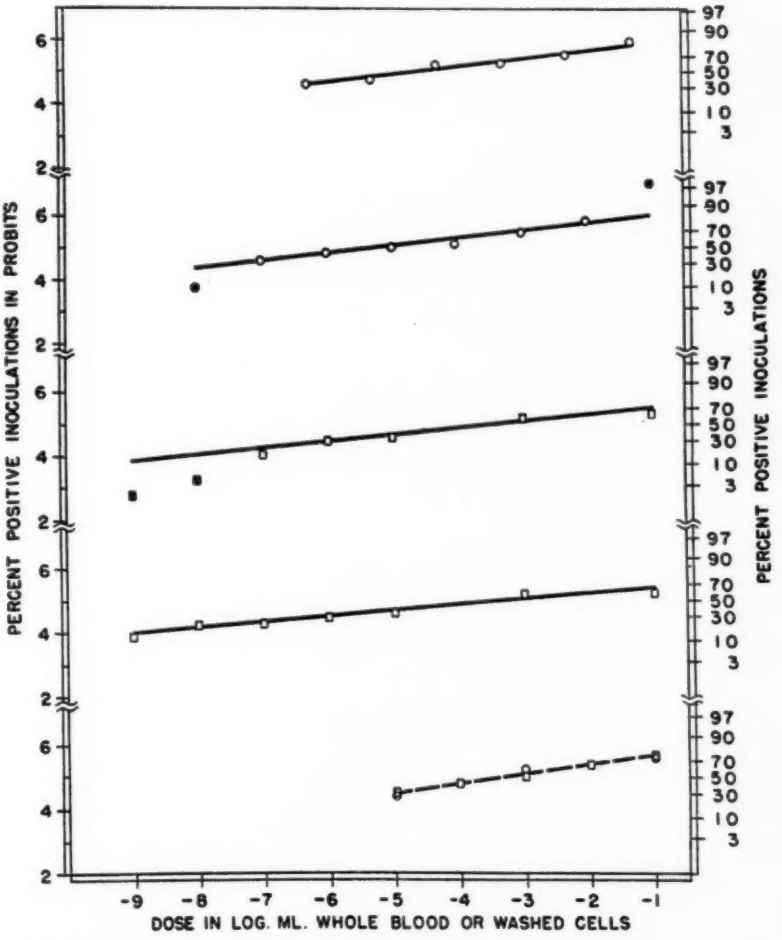
Results

In the studies on dose-response to the erythromyeloblastic leukosis virus (*5*), there were observed linear relationships between log dose of virus and both latent period $\left(\frac{100}{\text{latent period}}\right)$ and incidence of the disease converted to probits. It was the intention, initially, to look for similar relations in dose-response to materials containing the primitive cells. Actually, such was found to be the case, also, with cells; but, because of factors to be described in another publication, the present analysis is concerned only with the relation of incidence to dosage.

The data of the experiments essential for the analysis are recorded in table 1. Here, there are given the number of dose-groups in each experiment and the inoculums in terms of ml. of whole blood or ml. of cell suspension reconstituted to the relative volume of the whole blood from which the cells were derived. The corrected total number of chicks for each dose-group was calculated as described in an earlier report (*5*). There are shown also the number of chicks positive and the percent of positive inoculations. The variation in the number of chicks for the different dose-groups was due to the need for keeping the total to about 720 for each experiment, a number compatible with efficient handling. Larger numbers were used in the higher dilutions of experiments 53 and 56, in order to provide more accurate data in the region of low incidence.

Inspection of the data of table 1 reveals a definite indication of a dependence of incidence of positive inoculation on the dose of whole blood or of washed cells. When these results are analyzed on the basis of the relation of percent incidence to log dosage, the resulting regression is essentially linear, an observation like that made with the virus (*5*). Consequently, a graphic representation of the data with cells would not be unlike that with the findings on virus inoculation which have been shown in comparison with the sigmoid-incidence curves obtained in host-response to other viruses and materials in text-figure 1 of (*5*).

However, because of the uncertainty of the possible characters of the extremities of the percent-incidence curves, resort was had in this present work, as before, to transformation of percent incidence to probits. Such conversion, carried out in a manner identical with that used (*5*) with the virus data, results in the points indicated for each experiment in text-figure 1. The alignment of the respective groups of points relating probits to dosage in ml. of inoculum strongly suggests, with few exceptions, a linear relation, and this is borne out in the quantitative analysis of the data which yielded the lines drawn through the experimental points. The statistics derived through the appropriate calculations are given in table 2.



TEXT-FIGURE 1.—The relation of incidence, expressed in probits, to the logarithm of dosage in ml. of whole blood or washed-cell suspension. The experiments in order from above downward are 13 and 49, whole blood; 53 and 56, washed cells; and 51, both whole blood and washed cells. The data indicated by the closed circles and squares were omitted from the calculation of the respective regression lines.

In three of the experiments, 13, 51, and 56, all of the points may be regarded as falling significantly within the linear regression. The exceptions are seen in experiments 49 and 53. Two points of experiment 49, one at either extreme, obviously are outside the limits of variation established by the intervening points. Significant digression occurs in two points in experiment 53, both, in this instance, at one extreme, that in the region of high dilutions. These points in the two experiments were

TABLE 2.—*Data of experiments on host-response to whole blood and washed cells in erythromyeloblastic leukosis*

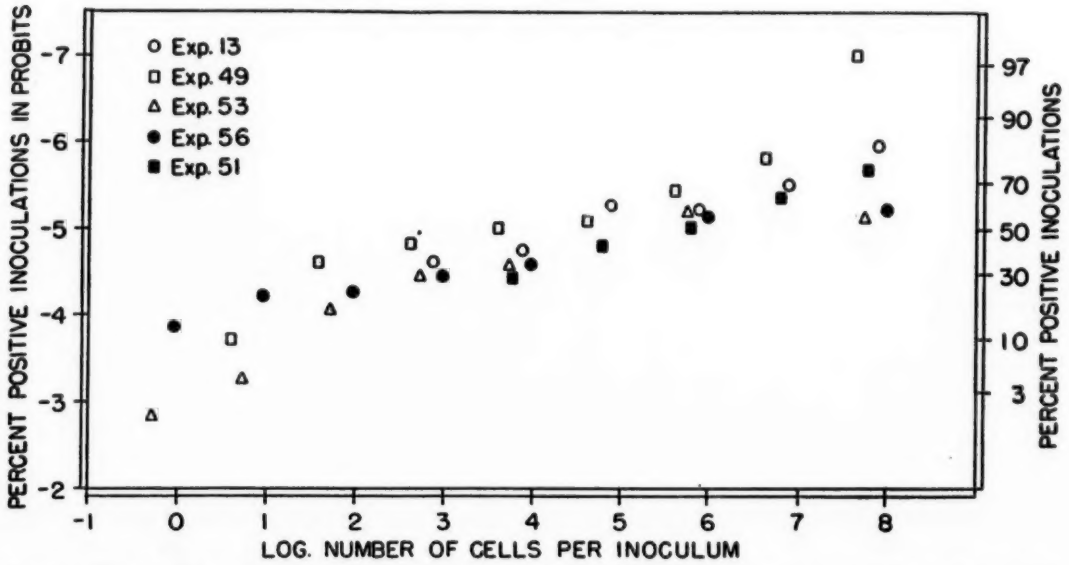
Inoculum	Experiment No.					
	13	49	53	56	51	
	Whole blood	Whole blood	Washed cells	Washed cells	Whole blood	Washed cells
Mean log dose, $\overline{X}$	−3. 934	−4. 541	−4. 540	−5. 679	−3. 063	−2. 991
Mean probit response, $\overline{Y}$	5. 189	5. 121	4. 654	4. 522	5. 070	5. 076
χ^2 for test for heterogeneity	4. 92	2. 13	7. 04	3. 17	2. 415	. 209
Degrees of freedom, d.f.	4	4	3	5	1	3
5 percent level for heterogeneity test	9. 5	9. 5	7. 8	11. 1	3. 8	7. 8
Slope of the regression, b	. 251	. 227	. 183	. 171	. 283	. 302
Standard error of slope	. 029	. 033	. 029	. 020	. 051	. 045
50 percent transmission dose, ID_{50}	−4. 687	−5. 077	−2. 651	−2. 888	−3. 311	−3. 177
Standard error of ID_{50}	. 210	. 257	. 451	. 556	. 285	. 207
Standard deviation, $\lambda=\frac{1}{b}$	3. 984	4. 412	5. 464	5. 840	3. 528	3. 312
Primitive cells per mm.^{3} × 10^5	15. 9	4. 3	5. 8	9. 5	6. 0	6. 3
Primitive cells per ID_{50}	33, 000	3, 600	1, 200, 000	1, 230, 000	293, 000	419, 000

omitted for the calculations locating the straight line. The possible basis for these considerable variations will be the subject of later discussion.

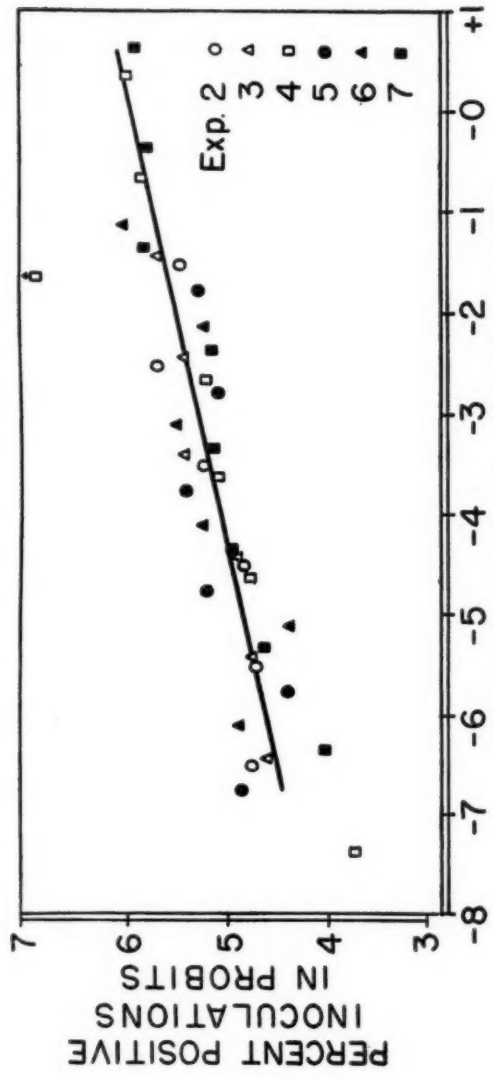
The relations shown in text-figure 1 all pertain to host-response to inoculums in terms of volume of whole blood or of the whole blood yielding the washed primitive cells. Primitive-cell counts on the inoculated materials yielded the values given in table 2. When the data were recalculated on the basis of dosage in numbers of cells contained in each inoculum, the relations shown in text-figure 2 were obtained.

The comparison of the transmitting potentialities of the cells with respect to number per inoculum has been given in table 2 at the level of the 50 percent point, and the values are seen to vary from 3,600 to 1,230,000. Another comparison is shown also in table 2 expressed as the dilution of the whole blood or cell suspension effective in inducing disease in 50 percent of the chicks inoculated.

The data of experiments 2 to 7 are grouped in text-figure 3 to show the evidence of trend of the responses toward a linear regression despite the small numbers of test animals employed. Grouping was accomplished by first calculating the regressions of probits on dosage in the individual experiments. The common regression was then calculated on the basis of the combined data of the individual experiments. Finally, the means of the individual regressions were adjusted to fall on the common regression. Using these adjusted values of the doses, which in the manipulation attained values greater or less than the actual dose volumes, the common regression line was recalculated and the adjusted points plotted as seen in text-figure 3. It is evident that variation of the individual points about



TEXT-FIGURE 2.—The relation of incidence, expressed in probits, to the logarithm of dosage in cells per inoculum calculated from the cell-counts made on the undiluted blood and cell suspensions given in table 2.



TEXT-FIGURE 3.—The accumulated data of experiments 2–7.

the line is very great because of the use of only 20 chicks for each point; nevertheless, there is no evidence of a systematic deviation of the points from the linear regression.

Discussion

The chief purpose of the present work has been to determine the character of host-response to inoculums containing the primitive cells occurring in the blood circulation of chicks with erythromyeloblastic leukosis. This was greatly desirable, for one reason, to learn whether the response to the cells was the same as or different from that to the virus and, for another, to devise a procedure suitable for bioassay of the transmission potentials of the primitive cells.

The results of the work have revealed, with the exception of certain deviant points, a linear relation between the incidence expressed in probits and the logarithm of the dose of whole blood or washed-cell suspensions. It is thus evident that, within a wide range of dosage in a given experiment, host-response was directly dependent on the dose inoculated. This finding is entirely similar to that encountered in experiments on host-response to the virus. A further similarity is seen in comparable values of the slopes of the respective regression lines obtained with the material containing cells here and with the virus in the previous work. It seems evident that this type of study and analysis gives promise of a usefulness in the bioassay of host-response to whole blood or cell suspensions equal to that observed in repeated experiments in the titration of the leukosis virus. In both instances the shallowness of slope, indicative of an exceedingly wide distribution in the individual susceptibilities of the host population, greatly complicates and restricts the work because of the large number of test hosts necessary for quantitative work.

Inspection of the values of table 2 and the individual points of the various experiments plotted to show the relation of response to dose in log number of cells (text-figure 2) indicates a considerable variation in the quantitative aspects of the responses in the respective experiments. This is evident from differences in the slopes of the lines, as well as in the levels of the responses. While differences in the responses seem obvious, no accurate method is available for comparing the transmission potentials of the various preparations quantitatively. Calculations have been made of the number of cells present in the inoculum inducing the disease at the 50 percent point level of incidence, though it is realized that the values thus derived may be of little, if any, significance. At this point the number of cells in the effective dose varied through a 341-fold range from 3,600 to 1,230,000 cells. It should be noted at once, however, that many factors may have contributed to whatever variation actually existed. Among these is an undoubted variation in the test-host population which might exert the principal and, indeed, perhaps the major influence on the apparent potency level. One known probable factor, which could affect the host population over and above natural variation, is a seasonal effect on response for which there is definite evidence. All of these factors, therefore, are completely separate from those pertaining to the cells *per se*.

From the point of view of the cells, it is clear that variation may occur in the genetic compatibility between the cells from various donor pools and the test hosts. It is seen that among all these possible fluctuations, it would be impossible, in experiments of this type, to sort out the criteria suitable for measuring those properties of the cells influencing their transplantability. It is interesting to see in table 1 and in text-figure 2 that despite the wide variation in the number of the cells associated with an incidence of 50 percent positive, the incidence observed in the highest dilutions in some of the experiments was far greater than that which might be expected on the basis of these apparent differences in the potencies of the various preparations. For example in experiment 56 the incidence with 10^{-9} ml. of washed-cell suspension was 13.45. The dose here was, according to the dilution factor, 1.59 cells. In experiment 53, 0.58 cells per inoculum induced an incidence of 1.68 percent and 5.8 cells brought down 4.24 percent of the chicks. An incidence of 10.34 percent occurred in experiment 49 with doses of 4.3 cells. These results give no evidence of a 341-fold difference (table 2) between the capacity of the cells of experiment 49 and that of the cells of experiment 56 to induce the disease. Instead, it would appear that the individual cells from both preparations were approximately equal in their ability to get along and multiply in the most susceptible hosts. Such an interpretation, however, is to be approached with caution because of the relatively high variability of the results in the region of the smallest dose. Further information clearly is obtainable by study of the cells from individual hosts under various conditions inoculated into samples of the same host populations. For such studies this form of leukosis affords unusual opportunities for the investigation of the factors governing transplantation, since the individual malignant cell can be counted with high accuracy, and any number of new strains of cells, on the basis of differences between the cells of different hosts, can be created simply by virus inoculation of the desired number of new hosts.

An unequivocal explanation of the basis for the deviations of host-responses at the extremes of some of the linear regressions has not been clearly established. Data bearing on certain of the possibilities, and at present in the process of analysis, provide indications of some of the factors concerned. It would appear that the final pattern of response to inoculations of the primitive cells is not characterized by an invariable state of host susceptibility and resistance at the level existing at the moment of inoculation, but by a summation of a series of continually changing responses associated with progressive alteration in this state during the period chosen for measuring the response. In this respect it seems possible that the characters of the response of chickens to these cells may be similar to those (*13*) of the response of rabbits to the papilloma virus. Evidence suggesting and substantiating this possibility will be presented in a forthcoming report.

The foregoing discussion is based on the tacit, if not the actual, assumption that the observed responses were related entirely to the cells of the blood and washed-cell inoculums. A question of immediate concern in the present work, however, is the identity of the inciting agent or material in the inoculums which induced the observed host-response. In the studies on virus, the material was filtered, and it is reasonable to conclude that the response obtained could be associated directly with the virus particles. Such cannot be the case in this work with cells; when whole blood was injected, plasma containing virus accompanied the cells in amounts not greatly different from

those employed in the experiments with filtered plasma; and in the instance of the washed cells, it would be overly optimistic to conclude that the washing process did more than remove most of the virus not attached or otherwise, possibly, associated with the cells. Consequently, it is obvious that both virus and cells entered the host concurrently. This is the more obvious when it is recognized that the cells contain virus. Administration of the cells, really, amounted to nothing different from homologous transplantation of the individual units of a malignant tumor which, undoubtedly, multiplied in certain instances in the new host. This differs wholly from the conditions associated with inoculation with virus alone, in which instance the primitive cells characterizing the disease must have arisen only from the tissues of the new host. If virus is present likewise in the cell inoculums, as it certainly was, then transmission by whole blood or washed-cell inoculums could be, if it actually did not occur, due to transplantation of malignant cells plus the activity of the virus on the tissues of the injected host.

With these possibilities in mind, it is of much interest to learn that host-response to the cells was in no way qualitatively different from that to the virus, within the limits of the doses employed, and, in addition, that the quantitative differences between the response to virus and that to the cells were of such magnitude that they can be expressed only statistically. In many experiments it has been clear that inoculums containing primitive cells usually, but not always, induce a higher incidence than those containing only virus, so that on the average the materials containing cells may be regarded as more potent than the virus in transmitting the disease. Overlapping of the responses to cells and those to the virus is such, however, that the findings of a single experiment cannot be recognized as due to cells or to virus. It should be emphasized at this point that this discussion is based on results obtained following inoculation of comparable volumes of filtered plasma and whole blood or washed cells, without regard to the number of virus particles or of individual cells concerned. It is evident, then, that no statements can be made about the relative potencies of cells and virus in the induction of erythromyeloblastic leukosis until the transmitting doses of the two materials can be compared on the basis of the number of individual units involved. From the knowledge of the extreme variations in the infectious capacity of plasma from individual chicks (*8*) and the number of virus particles in the plasma, it seems likely that, in the main, the cell unit is far more effective than that of the virus in transmission. It is possible that great variation will be found in the potentialities of cells from individual donors comparable with those of the virus, but no evidence for it has yet been seen. Whatever future work may reveal, it is evident that the nature or characters of the response to cell-containing inoculums in the present work provide no unequivocal evidence to discriminate between the possible activity of either virus or cells and, for that matter, to indicate that the cells themselves played any part whatever in the induction of the process.

Other evidence, however, suggests that the principal and, possibly, essentially all of the impact on the host of inoculums containing cells, under the conditions of the present work, was due to the cells. Indications of this are seen in experiment 51 in which the response to washed cells was identical with that to whole blood. This is based on the fact that free virus in the plasma of the whole blood from which the washed cells were obtained was diluted far beyond the concentration necessary for the production by virus of the response seen in this experiment. To conclude that the response was due to virus, therefore, would make necessary entirely unfounded assumptions about virus associated with the cells and free to act on the host after inoculation. It is notable that the observation that the whole blood of experiments 13 and 49 which contained both virus and cells seemed far more effective in the induction of the disease than the washed cells of experiments 53 and 56 cannot be interpreted as due to the activity of the virus of the plasmas of experiments 13 and 49. It happened that experiments 53 and 56 were made at a season of the year in which host-response was far lower than at other times. This seasonal variation in host-response will be taken up in a separate report.

Other information also of possible use in determining the respective roles of virus and cells in the response might be found in the latent-period data. Subsequent to the previous report (*5*) of studies on response to the virus, certain obstacles have been found to interfere with the usefulness of latent-period analyses in experiments such as those with the virus and with the cells. This will be the subject of another paper, but for the present purpose it may be stated that the latent period observed with a given volume of whole blood or washed cells, say 0.1 ml., is definitely much shorter than that seen with the amount of filtered plasma from the same volume of whole blood, or of the whole blood from which the cells were derived. This provides the only outstanding quantitative difference between the host-responses to the two materials.

Another means for detecting concerted cell and virus activity in preparations in which both are present might conceivably be found in the latent-period data on the following basis. It would appear that the distribution of resistance or susceptibility of the chicken population under study was approximately the same with respect to incidence for both cells and virus. In a selected instance, for example, cells might bring down 70 percent of the chicks with a 0.1 ml. dose of whole blood, and the same dose of the plasma of the same whole blood might result in an incidence of, say, 70 percent. If whole blood is now inoculated, both cells and virus possess, potentially, equal capacities to transmit the disease to approximately the same *number* of hosts but not necessarily the *same* individuals, and in each case about the same number of hosts, 30, would be left over unaffected by cells on the one hand and virus on the other. Inasmuch as the latent period of cell activity is definitely shorter than that of the virus, it might be expected that the pattern of the cell impression should be discernible well ahead of that of the virus. Therefore, if incidence is plotted against latent period, there might be expected a disruption of the curve of incidence distribution with respect to time due to the delayed induction of disease by virus in some of those 30 chicks unaffected by the cells alone. Thus it is conceivable that 70 percent of the 30, or 21, chickens might come down by direct virus infection after the principal effect of the cells had subsided. It is of considerable interest that no evidence of such a phenomenon has been observed, though sought repeatedly as will be described in a subsequent paper.

It is evident from this discussion that while host-response to the virus alone (in filtered, cell-free preparations) can be determined without doubt, the respective participations of cells and virus in preparations of whole blood or cells cannot yet be described. An outstanding feature of these studies on virus and cell preparations is the similar characters of the respective host-responses. From the discussion above it seems possible, though not definitely shown, that the same chickens which are susceptible (or resistant) to this virus are likewise susceptible to the cells and vice versa. It would seem that the principal difference between the two responses is a more rapid increase of the primitive cells to the diagnostic point following administration of the actual cells than of the cells arising from the host as the result of infection with the virus. An obvious difference exists between the factors which govern host-response to this leukosis virus and those influencing reponse to many other viruses which are not neoplastic. In the former instance, the host must be compatible not only with the virus but with the malignant cells derived through the exercise of the infectious process, while in the latter it seems reasonable to suppose that resistance would be directed toward the virus alone, rather than to the infectious process developed. That incompatibilities exist between host and these malignant cells arising from its own tissues is evident in fairly frequent retrogressions of the neoplastic process induced by the virus, as in other such processes not known to be of viral origin.

If it can be assumed that these diagnosable retrogressions, as well as others which probably never reached the diagnostic level, are related to incompatibility of host and cell, then it seems rather strange that the hosts are just as tolerant to malignant cells derived from other chicks as those from their own tissues. These considerations raise the question of whether the apparent host-response to virus is a response to the virus itself or whether the pattern is determined by the attitude of the host toward the malignant cells of its own tissues. If, however, the resistance is directed toward the virus entity and this resistance is not different qualitatively or quantitatively from that to the cells, there seems some reason to speculate that the virus and the neoplastic cells, which are chicken tissue, may possess a common biological component. While there is no unequivocal evidence that any other virus and host tissue share such a component, such may be the case in the instance of the leukosis virus.

Summary

Studies have been made of dose-response relations in the experimental transmission of avian erythromyeloblastic leukosis by whole blood and by the washed primitive cells present in the blood of chicks with leukosis. There was observed a linear relationship between the induced incidence of the disease transformed to probits and the log dose of the preparations containing the cells. This relationship, which is similar to that seen in previous studies on transmission by the virus present in filtered plasma, would appear to be suitable for bioassay of the transmission capacities of preparations containing cells, as are the analogous relations in the measurements on the infectivity of the virus.

Appendix

By B. R. Burmester

Histopathology of the Strain of Avian Leukosis under Investigation at Duke University

The principal pathologic manifestations of this strain of leukosis [Beltsville strain A (7)] have been studied and have been recorded here so that strains having similar or different pathologic characteristics may be compared with respect to their etiologic, immunologic, and transmission characteristics.

The chickens for this study were selected from several experiments and had been inoculated at 3 days of age with filtered plasma, whole blood, or washed-cell suspension. They were killed by exsanguination after having shown a distinct leukemia. However, the severity of the disease, as indicated by the number of immature cells in the circulation, varied widely. Specimens of the liver, heart, spleen, kidney, and bone marrow were fixed in 10 percent formalin or Zenker's formol-fixative fluid for microscopic study. Tissue-touch preparations of the liver, spleen, and bone marrow, as well as blood smears for special staining, were fixed in methanol. All tissue-touch preparations and some blood smears were

treated with May-Grünwald's stain and Giemsa's stain. Wright's stain was used on all other blood smears. Tissue sections were stained with Ehrlich's hematoxylin and counterstained with triosin. A second section of each tissue was counterstained with azure II-eosin or tetrachrome stain.

The chief, and in many cases the only, pathologic alteration occurred in the blood and bone marrow. The blood change was dominated by the appearance of large numbers of medium to large mononuclear, agranular basophilic cells with few, or no, differentiating characteristics. However, in a few birds of certain passages, myelocytes were present in significant numbers, and most of them were early forms containing basophilic granules, though in some cases there were many of the more mature forms of myelocytes containing eosinophilic rods. Leukemic cells in the peripheral circulation in some of the advanced cases made up as much as 60 percent of all blood cells. The anemia which was present in such cases appeared to be due to a crowding out of erythrocytes by the large number of neoplastic cells in the bone marrow and the blood stream. None of the blood smears examined showed evidence of erythroid stimulation. Only a few polychromatic erythrocytes were seen in some blood samples, and occasionally an erythroblast was found.

The bone marrow was invariably hyperplastic. The yellow marrow of the long bone was entirely replaced by a very soft, dark-red marrow having a smoky appearance. Most of the marrow was made up of centers of proliferating hemocytoblasts or early myeloblasts, with the result that the sinuses were reduced to narrow spaces. Only a few differentiated forms of either the myeloid or erythroid series were present in the marrow. The proportion of various cells in the sinuses was similar to that found in the circulating blood.

Changes in the visceral organs were variable and were correlated with the extent and duration of the leukemic condition. Early or mild cases showed no gross changes, although large numbers of leukemic cells were present in the vascular bed of all organs examined. In advanced cases, the liver was usually enlarged and either dark-red or grayish in color, with or without isolated light-gray areas. Enlarged livers showed a marked distention of the capillaries and sinusoids with leukemic blood. In some cases there was atrophy of the hepatic cords, allowing further distention of the sinusoids. Many livers had extensive perivascular accumulations of primarily the same undifferentiated leukemic cells as those seen in the circulation, together with myelocytes. Sections of the heart, kidney, and spleen of advanced cases usually showed extensive leukostasis. In addition, in some cases the myocardium and parenchyma of the kidney, and in many cases the spleen, were infiltered with the leukemic cells.

With the exception of certain variations related to the relative proportion of myelocytes in the leukemic-blood picture, the pathologic changes noted in the cases studied were similar to those reported by Ellermann (*14*), Engelbreth-Holm (*15*), and Nyfeldt (*16*), except that Nyfeldt did not find the condition of leukostasis associated with myeloblastosis.

As previously indicated, the dominant cell-type was a large, mononuclear, basophilic cell without differentiating characteristics. On the basis of its morphology and staining characteristics it may be tentatively classified as a stem cell or a hemocytoblast. However, since a large number of myelocytes were found in the circulating blood of many cases and in almost all tissue-infiltration areas, it may be inferred that these stem cells were early myeloblasts or hemocytoblasts having the ability to differentiate into myelocytes. The absence of significant numbers of polychrome erythrocytes or erythroblasts in the circulating blood, and their presence in the bone marrow in relatively few numbers, indicated that the erythroid series was not involved. Thus, it would appear that the virus of this strain has the particular property of invoking the neoplastic process in the early myeloblasts without any tendency to affect the closely related erythroblasts or the multipotent hemocytoblasts. Since the pathologic manifestations of this strain have remained consistent through the various passages of the 56 experiments shown in Chart 1 of (*5*), Chart 1 of (*8*) and Chart 1 of the present report, it appears to be a relatively pure strain of myeloblastosis.

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The Cytological Effect of Chemicals on Ascites Sarcomas. I. Partial Damage in Tumor Cells by Podophyllin, Followed by Temporary Regression and Prolongation of Life of Tumor-Bearing Rats [1,2]

SAJIRO MAKINO *and* TATSUYA TANAKA,[3] *Zoological Institute, Hokkaido University, Sapporo, Japan*

Podophyllin, a resin derived from the roots and rhizomes of the May-apple plant (*Podophyllum peltatum*), contains podophyllic acid, podophyllotoxin, picropodophyllin and other compounds (*17*, *3*). Recently, podophyllin attracted the attention of biologists when a paper published by Kaplan (*5*) described a striking effect of this substance in reducing venereal warts. King and Sullivan (*6*, *7*, *19*) later pointed out that podophyllin and colchicine possess similar properties in respect to the treatment of warts. Sullivan and Wechsler (*18*), and Sullivan (*20*) found podophyllin to be effective as a mitotic poison in plant cells. This background naturally excited interest in the possible effects of podophyllin on growth processes of tumors. More recently, Shear and his associates (*1*, *4*, *9–11*, *23*, *24*), and Ormsbee, Cornman, and Berger (*16*, *17*) described the effects of this drug on tumors. Cornman and Cornman (*3*) showed quantitatively that the effects of podophyllin and of colchicine are essentially the same in respect to division of normal cells. Most of these studies deal, however, with the histological changes of the tumor tissue or the decrease in mitoses in the tumor after exposure to the drug. It is now desirable to study both histological and karyological changes in tumor tissues treated with chemicals in analyzing tumor damage or regression.

The present paper is concerned with the action of podophyllin on cell division, and its influence on growth of ascites tumors.

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Materials and Methods

The following experiments employed both the Yoshida sarcoma and the MTK-sarcoma I, carried in Wistar rats. These ascites tumors, which are induced transplantable fluid sarcomas, offer a number of advantages and are well suited for the purposes of this type of study (*13, 14*).

Intraperitoneal injection of 1 cc. of aqueous solution of crude podophyllin without exception killed the tumor-bearing animal within a few hours after treatment, despite the use of such low concentration as 0.01 percent. After several tests, it was found that podophyllin dissolved in isotonic glucose solution exerted a selective effect on tumor cells. The range of concentrations of podophyllin used was 0.1, 0.05, and 0.01 percent. Tumor-bearing rats could not survive after injections of podophyllin in concentrations higher than 0.1 percent.[4] To prepare 0.1 percent solution of podophyllin, 0.1 gm. of podophyllin was dissolved in 100 cc. of isotonic glucose solution (6.73 gm. of glucose). One cc. of this podophyllin solution per 100 gm. of body weight was injected intraperitoneally into tumor-bearing rats on the 6th day after transplantation of the tumor, and again on the 13th and 20th days. By this time, the most active proliferation of the tumor cells had been attained in the peritoneal cavity of the animal, and the tumor was in an advanced stage of development (*15, 21*). All experiments were run at room temperature.

Smear preparations were made from a uniform droplet of the tumor ascites obtained by abdominal puncture at appropriate intervals following the application of the chemical. The slides were colored with aceto-carmine or acetic gentian violet. Giemsa's preparations were also studied for comparison (*12, 14*).

Results

The Yoshida sarcoma and MTK-sarcoma I proved to respond similarly to podophyllin. Hence, the following description applies to both cases. The description was based on the results obtained from eight experimental animals in comparison with results from the same number of untreated tumor-bearing animals used as controls.

Following injection of 0.01 percent solution of podophyllin, the mitoses of tumor cells were arrested at metaphase. The chromosomes agglutinated into several clumps, and nuclear disintegration followed. But after treatment with this concentration of the drug, the number of affected cells was rather small. Higher concentrations, such as 0.05 and 0.1 percent, evoked similar responses and consistently produced greater damage to tumor cells. The following descriptions are chiefly based on the results produced by application of a 0.1 percent solution of podophyllin.

Induced Karyological Changes Leading to Degeneration of Tumor Cells

Ten to 15 minutes after injection of 0.1 percent podophyllin solution, the ascites tumor displayed a large number of tumor cells arrested at th stage of metaphase. The majority of affected cells were of relativ el large size, and the chromosomes were irregularly condensed and scattere in the cell without any evidence of equatorial arrangement. At a late

[4] These figures designate the concentrations of podophyllin at the time of preparation only. They do not indicate the dose with which the tumor cells are affected in the peritoneal cavity, because the dose by the intraperitoneal route of administration is unknown at present.

time following injection of the chemical, the chromosomes were shorter and more irregularly thickened, and at a still later stage they appeared as unusually deformed, rounded or bizarre bodies. At 30 to 40 minutes after injection, almost all cells at metaphase showed chromosome clumping, or further advanced degenerative changes. By this time, the cytoplasm seemed to be affected; it showed irregular processes or atypical ameboid protrusions resembling pseudopodia. The cells remaining at the stage of prophase exhibited no visible change through these periods of time, and there was no change in the relative number of prophase cells. In striking contrast, there was a pronounced decrease of the number of cells in anaphase. The data presented in table 1 illustrate this feature clearly.

TABLE 1.—*Incidence of metaphase and anaphase stages in tumor cells on the 6th day after transplantation of the Yoshida sarcoma*

Stage	Control	0.1 percent podophyllin Time after injection	
		20 min.	30 min.
Metaphase	192 (68.6%)	140 (92.1%)	306 (97.5%)
Anaphase	88 (31.4%)	12 (7.9%)	8 (2.5%)
Total	280	152	314

By 60 minutes or more after injection, the disintegration of tumor cells was more marked; some of the condensed chromosomes were agglutinated into irregular masses of different size and shape distributed about the cell. In some instances, these masses were stuck together, producing pyknotic aggregations of chromatin; death of the cell probably resulted in these cases. In figures 1 to 9, a series of cells show the process of disintegration after treatment with podophyllin.

EFFECT OF PODOPHYLLIN ON THE GROWTH OF ASCITES SARCOMAS

Four to five hours after injection of podophyllin, large numbers of damaged cells (fig. 13) appeared in samples of the ascites tumor. Rough counts in this case showed that more than 80 percent of the tumor cells were undergoing degeneration. By 7 to 10 hours, only a few undamaged cells could be found (figs. 14 and 15). After this time, samples of the ascites tumor contained large numbers of eosinophilic and neutrophilic leukocytes (figs. 16 and 17). Lymphocytes were few in number. In preparations obtained 18 to 24 hours after treatment, most of the debris from degenerating cells had disappeared from the ascites, due probably to liquefaction and transportation, or to phagocytosis by leukocytes (figs. 18, 19). At 7 to 10 hours following injection, the ascites was considerably reduced. Figure 12 shows the number of tumor cells observed in a sample just before the injection of podophyllin (on the 6th day after transplantation), while figures 18 and 19 show samples of cells in the ascites at 24

hours after the injection of podophyllin. It is evident from these figures that the tumor regressed temporarily in podophyllin-treated animals and the regression is very striking in comparison to the untreated tumors. Particularly important and noticeable in these figures is the fact that interspersed among the eosinophilic and neutrophilic leukocytes, there are a certain number of tumor cells of extremely small size, which have persisted without being visibly damaged by the drug. These cells are characterized by a small amount of cytoplasm and a single, well-defined, compact nucleus showing distinct nucleoli (figs. 18 and 19). Further description of these resting tumor cells will be given in the next section.

No harmful effect on the animal was observed with the maximal concentration of 1 cc. of 0.1% solution per 100 gm. of body weight.

The Presence of the Tumor Stem-Cells[5] and Their Behavior in Response to Podophyllin

Based on the results of cytological studies on the Yoshida ascites sarcoma and the MTK-sarcomas of rats, Makino and his co-workers (*12–15, 21*) reached the conclusion that in these tumors a definite strain of tumor cells exists which has its own characteristic chromosome constitution and multiplies by regular mitosis. The individuality of the chromosomes in these tumor stem-cells remains unchanged during successive transplant generations, and the growth of the tumor depends primarily upon the proliferation of these stem-cells. The fate and behavior of the tumor stem-cells under the influence of podophyllin was thus considered.

At 18 to 24 hours after the injection of podophyllin, most of the tumor cells were damaged, while certain of them characterized by a small amount of cytoplasm and well-defined compact nuclei with distinct nucleoli remained unaltered (figs. 18 and 19). These small-sized resting tumor cells evidently divide at a later time, for at about 24 hours after injection division figures were few in number, and on the 3rd day were increased (figs. 20 and 21). Through the 4th to 6th day after injection, such cells appeared in active division (figs. 22, 23). The increase in the number of tumor cells paralleled an accumulation of tumor ascites, which caused expansion of the abdomen. A second injection of 0.1 percent podophyllin was given on the 7th day after the first injection, at which time the growth of the tumor was well advanced. There was again damage to tumor cells with consequent temporary regression of tumor growth, much as happened in the case of the first injection. By 5 days after the second injection, regrowth of the ascites tumor had been attained again in every rat with remarkable accumulation of tumor ascites. A third injection was followed by regression and subsequent regrowth. The life span of the triple-treated animals is shown in table 2. It is clear that the prolongation of life of tumor-bearing rats treated with podophyllin is very striking.

[5] The term "stem-cells" corresponds to the term "strain-cells" used in our previous papers (*12–15*).

Table 2.—*Life span of tumor-bearing animals following transplantation of ascites sarcomas (Y, Yoshida sarcoma; M, MTK-sarcoma) and after treatment with podophyllin*

	Untreated			Treated (1 cc./100 gm.) on 6th, 13th and 20th day		
Tumor	Sex	Body weight	Life span	Sex	Body weight	Life span
		(*grams*)	(*days*)		(*grams*)	(*days*)
Y	♀	100	14	♀	100	41
Y	♀	95	11	♀	100	42
Y	♂	100	11	♂	100	38
M	♀	95	10	♀	95	49
M	♂	95	11	♂	95	42
M	♂	90	10	♂	100	51
M	♂	95	11	♂	110	56
M	♂	90	13	♂	95	47

The Nature of the Small-Sized Tumor Cells Undamaged by Podophyllin

As mentioned above, these unaffected small tumor cells appeared in mitotic division at about 20 or more hours after any injection. They divided in a quite regular mitotic manner. Detailed observations revealed that the chromosome complex of these cells was very characteristic not only in respect to shape of chromosomes but also to number, and was essentially similar to that of other tumor stem-cells (*12*, *13*). The small-sized tumor cells of the Yoshida sarcoma possess a chromosome complex consisting of 22 to 24 rod-shaped elements and 16 to 18 J- and V-shaped ones (figs. 10 and 11). In the MTK-sarcoma I, two types of cells occur, differing in their chromosome complements. One of them contains 24 to 26 rod elements and from 15 to 17 J- and V-shaped ones, whereas in the other type of cell there are 23 to 25 rods and 16 to 17 J's and V's. Further, the numerical data obtained in the examination of preparations from 2 to 4 days after injection of podophyllin support this conclusion clearly by showing that almost all the dividing cells derive from the tumor stem-cells, as judged by the existence of approximately 40 chromosomes and their own characteristic chromosome constitution. In the Yoshida sarcoma, 2 days after injection, a count of 196 dividing stem-cells showed 84.7% with normal division, and 8.7% with sticky chromosomes, while 6.6% of the divisions were tri- or tetrapolar. In 348 dividing stem-cells of the MTK-sarcoma I, 72.1% were normal, 23.3% had sticky chromosomes, and 4.6% were tri- and tetrapolar.

Discussion

From the above evidence, it is apparent that the temporary regression in the growth of the tumor and the consequent prolongation of the life of the tumor-bearing animal are due to damage of a large number of tumor cells by the podophyllin. In this treatment, however, some of the tumor stem-cells remain unaffected by the action of podophyllin. These per-

sistent unaffected tumor stem-cells constitute the primary source of the subsequent growth of malignant cells, leading to the reappearance of the tumor in the treated animals. Podophyllin in the concentrations employed in this study failed to destroy these stem-cells, and thus no case of complete recovery from the disease was found in any tumor-bearing host. Higher concentrations of podophyllin or successively repeated treatments were lethal to the tumor-bearing animals. The present experiments succeeded in prolonging the life of the host but failed to effect complete recovery from the disease.

The reason that some of the tumor stem-cells were able to persist alive and unaffected by podophyllin, whereas most of the other tumor cells were damaged, is one of the important and fundamental problems of research on tumor cells. The nature of these stem-cells has been under study from both morphological and physiological standpoints. The above experiments merely show that the resting stem-cells are able to resist unfavorable conditions of the surrounding medium. Most probably, the resting stem-cells have acquired at their surface certain protective properties. In other words, by transforming into the small-sized, resistant form, the surface of these cells may become less permeable to noxious substances of the surrounding medium. Upon removal of the drug, they begin multiplication and serve as the primary source of regrowth of the malignant tumor. Therefore, complete recovery from the disease can be expected only with complete destruction of these resistant stem-cells. Before going further with this problem, an analysis of the physiological properties of this strain of cell would be highly desirable. But it may be worth recording that the present experiments have served to develop a procedure for the study of cancer with a hope toward prolonging the life of tumor-bearing animals by judicious use of podophyllin.

Damage to the tumor cells follows the pattern described for the effects of podophyllin. The cell is blocked at metaphase and then undergoes pyknotic degeneration. MacCardle (*9*) reported that podophyllin, given in low doses (20 micrograms) over a short period of time, induced changes in cells of Sarcoma 37 that are essentially identical to those induced by the methyl ether of acetyliodocolchinol. In treated cells, observed in the living state, many clear vacuoles develop among the chromosomes arrested in metaphase. These seem to coalesce into a single large sphere in the midplate region of the cell. A high dose of podophyllin induces stark necrosis at once, without preliminary distortion of mitotic figures. According to unpublished data cited in the paper by Cornman and Cornman (*3*), similar results have been obtained in some experiments with tumors of the mouse. Also, Sarcoma 180 was found to respond more visibly to crude podophyllin than to derivatives of the drug. Further, it was stated that injection of sublethal doses of podophyllin into mice bearing spontaneous mammary carcinoma (or five-day-old Sarcoma 180) resulted in a slowing of growth and even temporary regression of tumors in some cases.

Cornman and Cornman (*3*) have studied the effects of podophyllin on cell division in some marine eggs. Podophyllin derivatives tested by them produced mitotic effects similar to those induced by colchicine, showing that podophyllin derivatives cripple or destroy the achromatic figure. It may be said that a 0.1 percent solution of crude podophyllin with isotonic glucose solution injected into the peritoneal cavity of rats bearing ascites sarcoma acts as a mitotic poison, damaging the tumor cells at metaphase. In podophyllin-treated animals, cell division is arrested at metaphase and chromosomes transform into irregular masses after condensation. Then they stick

together in several chromatic clumps distributed throughout the cytoplasm. The cells die without advancing beyond metaphase. The condition here presented closely resembles that obtained by the use of large doses of colchicine or by long exposures to the drug; in these cases, the dividing nuclei not only fail to produce a spindle, but the chromatic material becomes pyknotic and degeneration of the cell follows (*8*). The effect of colchicine on the growth of the Yoshida sarcoma has been studied by certain authors (*22*); their results uniformly indicate that colchicine acts by arresting division of tumor cells with subsequent formation of mitotic abnormalities, and slight prolongation of life of tumor-bearing animals. Colchicine fails to eliminate the Yoshida tumor completely, however.

It was reported that cleavage of marine eggs poisoned by 0.01–0.02 mM colchicine was improved by 8–32 mM glucose, and that podophyllotoxin could also be partially neutralized by glucose at 16–64 mM/l (*2*). In the present study, podophyllin was prepared with isotonic glucose solution, which protected the animals against lethal doses of podophyllin. It is not known whether glucose can protect animals against the effects of overdosage of podophyllin.

Summary

It was found that podophyllin, a resin derived from the May-apple plant, acts as a mitotic poison at metaphase in tumor cells of the Yoshida ascites sarcoma and of the MTK-sarcoma I in Wistar white rats. A 0.1 percent solution of crude podophyllin prepared with isotonic glucose solution was injected intraperitoneally in the proportion of 1 cc. per 100 gm. of body weight of the tumor-bearing rat. In treated cells, the chromosomes were condensed into irregular bodies, and stuck together, forming several pyknotic clumps scattered throughout the cytoplasm. The cells evidently died without proceeding beyond metaphase; and this led to inhibition and temporary regression of the tumor. A few days later, the tumor began to grow, the renewed growth being caused by the proliferation of certain morphologically distinct tumor stem-cells which remained alive and undamaged by the drug. Three separate injections at intervals of 7 days in the same tumor-bearing animals produced a striking prolongation of the life span of the host.

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PLATES

PLATE 129

Photomicrographs of ascites tumor cells from podophyllin-treated tumor-bearing rats. Smear preparations. Aceto-carmine or acetic gentian violet stain.

FIGURE 1.—Abnormal condensation and irregular agglutination of chromosomes in Yoshida tumor cells, 15 minutes after injection of podophyllin. × 600

FIGURE 2.—Yoshida tumor cells from the same rat as in figure 1, 20 minutes after injection. × 600

FIGURE 3.—An affected tumor cell in metaphase, and an unaffected tumor cell in prophase, 20 minutes after injection. Yoshida tumor. × 600

FIGURE 4.—Advanced condensation and agglutination of chromosomes in metaphase cells of Yoshida tumor. A prophase cell with unaltered chromosomes, 30 minutes after injection. × 600

FIGURE 5.—Advanced condensation of chromosomes in metaphase cells with evidence of disintegration of cells, 3 hours after injection. MTK-sarcoma I. × 600

FIGURE 6.—Advanced condensation of chromosomes in metaphase cells with evidence of disintegration of cells, 4 hours after injection. MTK-sarcoma I. × 600

FIGURE 7.—Advanced condensation of chromosomes in metaphase cells with evidence of disintegration of cells, 4 hours after injection. MTK-sarcoma I. × 600

FIGURE 8.—Irregularly condensed chromosomes aggregated into pyknotic masses 4.5 to 5 hours after injection. MTK-sarcoma I. × 600

FIGURE 9.—Irregularly condensed chromosomes aggregated into pyknotic masses 4.5 to 5 hours after injection. MTK-sarcoma I. × 600

FIGURE 10.—Metaphase plate of a tumor stem-cell with subdiploid chromosomes, 4 days after injection. Yoshida sarcoma. × 600

FIGURE 11.—Yoshida tumor cells from same rat as in figure 10, after injection. × 1,200

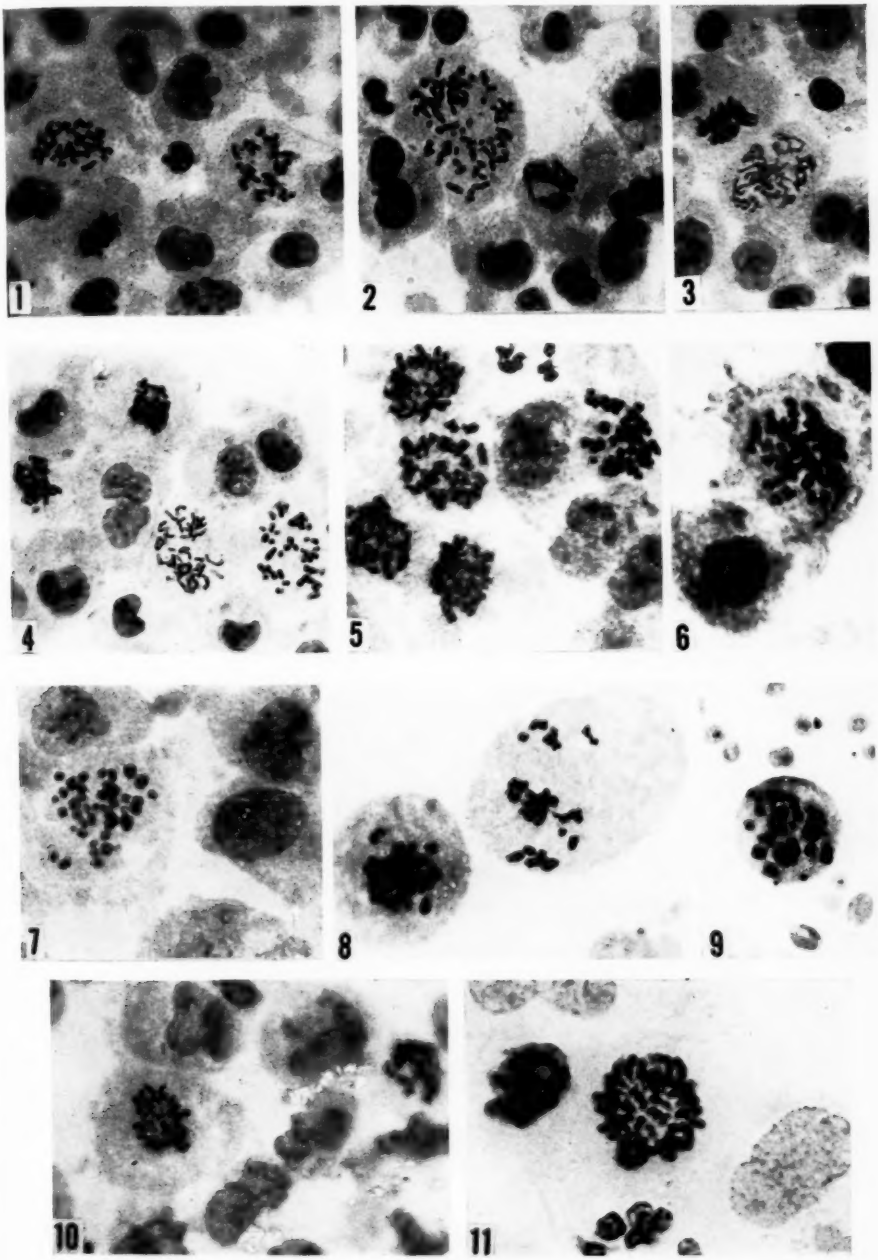

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PLATE 130

Photomicrographs of ascites tumor cells in rats. Smear preparations. Aceto-carmine or acetic gentian violet stain.

FIGURE 12.—Yoshida sarcoma cells on the 6th day after transplantation, just before podophyllin injection (control). × 400

FIGURE 13.—Disintegrative Yoshida sarcoma cells of podophyllin-treated rats. Note some small-sized tumor cells remaining unaffected. Five hours after injection. × 400

FIGURE 14.—Tumor cells from same animal as in figure 13, 10 hours after injection of podophyllin. × 400

FIGURE 15.—Tumor cells from same animal as in figure 13, 10 hours after injection of podophyllin. × 600

FIGURE 16.—Leukocytes in MTK-sarcoma I among affected and unaffected tumor cells, 15 hours after injection of podophyllin. × 400

FIGURE 17.—MTK-sarcoma I ascites cells observed 18 hours after injection of podophyllin. × 600

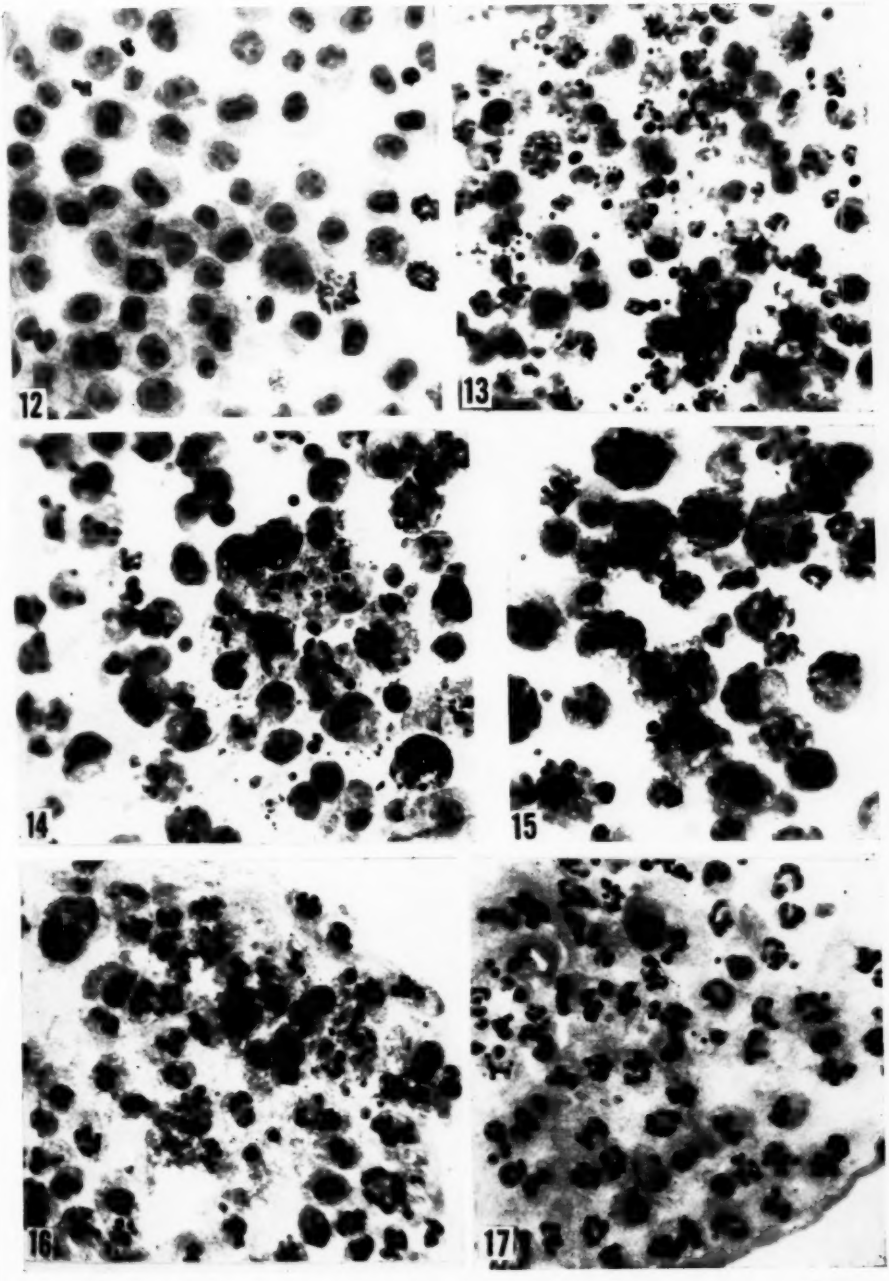
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PLATE 131

Photomicrographs of ascites tumor cells in rats, following treatment with podophyllin. Aceto-carmine or acetic gentian violet stain. × 400

FIGURE 18.—Yoshida sarcoma ascites cells at 24 hours after injection of podophyllin. Most of the destroyed tumor cells were apparently absorbed, and small-sized tumor cells (indicated by arrows) remain unaffected among leukocytes.

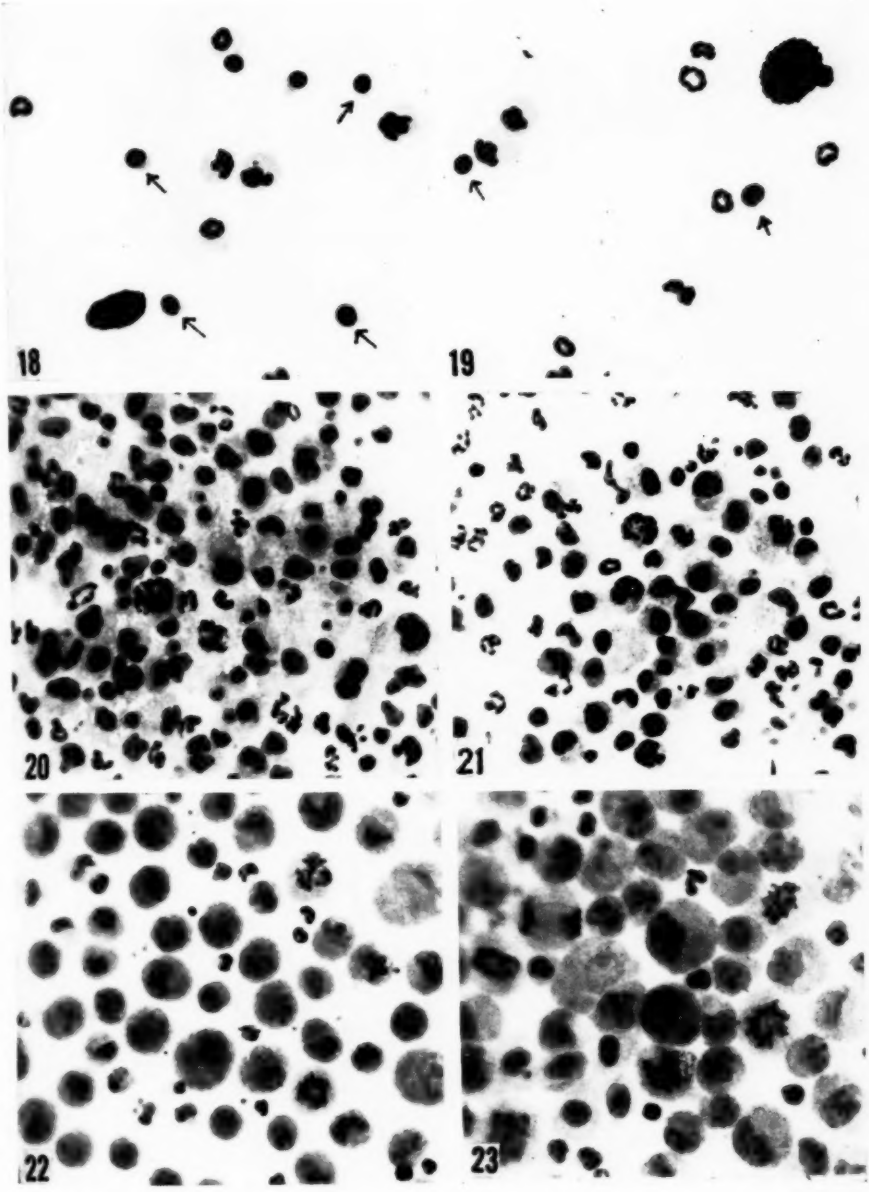
FIGURE 19.—Yoshida tumor cells from another rat at 24 hours after injection of podophyllin.

FIGURE 20.—Yoshida sarcoma ascites cells taken 3 days after injection of podophyllin, showing proliferative tumor cells.

FIGURE 21.—Yoshida sarcoma ascites cells from same animal as in figure 20, 3.5 days after injection.

FIGURE 22.—MTK-sarcoma I ascites cells observed 4 days after injection of podophyllin, showing many tumor cells. Many mitotic figures.

FIGURE 23.—MTK-sarcoma I ascites cells taken on the 6th day after injection, showing increase in tumor cells, and many dividing cells. The malignant growth of the tumor has reappeared. Compare this figure with figure 12 taken just before treatment.



239106—53——13

Damage Induced in Sarcoma 37 With Chemical Agents. V. Derivatives of Colchicine and Isocolchicine [1]

J. Leiter, J. L. Hartwell, G. E. Ullyot, *and* M. J. Shear, *Laboratory of Chemical Pharmacology, National Cancer Institute,*[2] *Bethesda, Md., and Smith, Kline and French Laboratories, Philadelphia, Pa.*

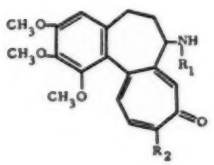
Two recent publications (*1*, *2*) reported that many derivatives of colchicine were capable of producing hemorrhage and necrosis in Sarcoma 37 after a single subcutaneous injection. These compounds were of three general types, *viz.*, derivatives of trimethylcolchicinic acid, of colchinol, and of colchiceinamide. Several of these compounds exhibited virtually the same tumor-damaging activity as colchicine against Sarcoma 37, but had greatly reduced lethality. Neither the vehicle employed, nor the solubility, appeared to be responsible for the differences in activity or toxicity displayed by these derivatives. Although some correlations between structural modifications and tumor-damaging activity could be drawn, no general relationship between potency and structure was clearly evident; in addition, some anomalous findings were made.

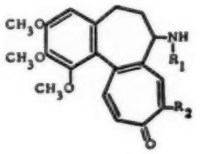
This paper gives the results obtained with other types of derivatives of colchicine, following subcutaneous injection into mice bearing Sarcoma 37. Of the 11 compounds reported here, 4 were *iso*-forms of known colchicine derivatives. After hydrolysis of colchicine to colchiceine, alkylation of the hydroxyl group of the C ring results in a mixture of two structurally isomeric alkyl ethers in which there is transposition of the alkoxyl and ketone groups of the C ring (text-fig. 1). Such compounds are designated, respectively, as *colchicine* and *isocolchicine* derivatives. Two of these *iso*-compounds (*isocolchicine*, NCI-2824, and *isocolchiceine ethyl ether*, NCI-2778) had been prepared and characterized by earlier investigators (*3*, *4*); these are the *iso*-analogs of *colchicine* and *colchiceine ethyl ether*, respectively. The other two *iso*-compounds are *isocolchiceinamide*, NCI-2798, and *isotrimethylcolchicinic acid methyl ether* d-*tartrate*, NCI-3126.

Seven other derivatives of colchicine were also examined. *Colchiceine ethyl ether*, NCI-2826, is the normal form of one of the *iso*-compounds mentioned above. *Hexahydrocolchicine*, NCI-1987, and *hexahydrocolchiceine*,

[1] Received for publication October 22, 1952.

[2] National Institutes of Health, Public Health Service, Federal Security Agency.



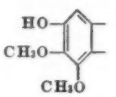


R_1	R_2	NORMAL FORM	ISO-FORM
CH_3CO	OH	---	COLCHICEINE (NCI - 1040)
CH_3CO	OCH_3	COLCHICINE (NCI - 1 v)	ISOCOLCHICINE (NCI - 2824)
CH_3CO	OC_2H_5	COLCHICEINE ETHYL ETHER (NCI - 2826)	ISOCOLCHICEINE ETHYL ETHER (NCI - 2778)
CH_3CO	NH_2	COLCHICEINAMIDE (NCI - 2177)	ISOCOLCHICEINAMIDE (NCI - 2798)
H	OCH_3	TRIMETHYLCOLCHICINIC ACID METHYL ETHER (NCI - 1136)	ISOTRIMETHYLCOLCHICINIC ACID METHYL ETHER (NCI - 3126)

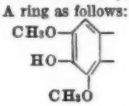
TEXT-FIGURE 1.—Normal and *iso*-derivatives of colchicine.

NCI-2496, are reduction products of colchicine and colchiceine, respectively, in which two of the double bonds and the carbonyl group of the C ring have been hydrogenated. *Desmethylcolchicine*, NCI-2825, which has the structure of colchicine with one of the methoxyl groups in the A ring demethylated, was isolated from U. S. P. colchicine by a chromatographic technique (*5*) and is probably identical with "Compound C" isolated from the colchicum plant by Šantavý (*4*). The position of the free hydroxyl group on the A ring has not yet been determined.[3] *Colchinoic acid*,[4] NCI-2497, its *methyl ester*,[5] NCI-2498, and *N-benzoylcol-*

[3] Since this manuscript was submitted for publication, Šantavý and Talaš (ŠANTAVÝ, F., and TALAŠ, M.: Látky ocúnu a jejich deriváty XXVIII. Příspěvek ke konstituci látek C a E_1. Chemické listy 46: 373–375, 1952) have determined the positions of the hydroxyl and the 2 methoxyl groups in the A ring to be as follows:



We presume that this is probably the structure of our desmethyl-compound. In the same publication, the authors have also determined the structure of another desmethyl-compound (Compound E_1), isolated from the colchicum plant, with the substituents on the A ring as follows:

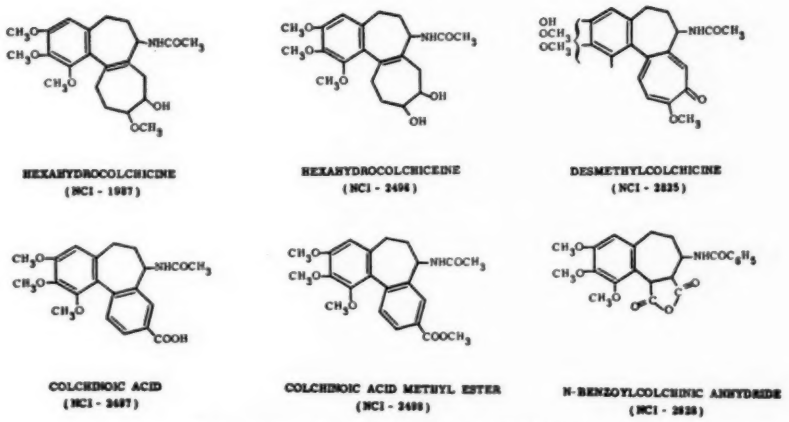


The biological data of Šantavý, Lang, and Malinsky (cited later) suggest that it is less toxic than Compound C but has about the same relative potency.

[4] We have named this compound as a derivative of *colchinol*. It has also been called *colchicic acid* by Šantavý and Reichstein (*4*), but this name had previously been used in 1888 by Zeisel (*7*) for another derivative, *viz.*, the completely demethylated compound from colchicine. Fernholz (*8*) has called this same compound *allocolchiceine* (see footnote 5). In view of the confusion in terminology, we prefer the name *colchinoic acid*, which properly notes it as a derivative of *colchinol*.

[5] This compound was synthesized by Šantavý (*6*) and named by him the *methyl ester* of *colchicic acid*. Fernholz (*8*) called it *allocolchicine* because of its isomerism with colchicine. For clarity and consistency, we prefer the name, *methyl ester* of *colchinoic acid*. In fact, Fernholz (*8*) demonstrated the relationship of these compounds to *colchinol* by conversion of *colchinoic acid* to *colchinol*.

chinic anhydride, NCI-2628, are degradation products which have been obtained from colchicine. Text-figures 1 and 2 give the formulas of the compounds used.



TEXT-FIGURE 2.—Derivatives of colchicine.

Materials and Methods

The compounds employed are listed in table 1, together with the code numbers and melting points. The compounds that were not sufficiently soluble in an aqueous vehicle were injected as suspensions in olive oil. The vehicles employed with the individual compounds are listed in tables 2 and 3. The procedure for evaluating potency in damaging Sarcoma 37 was similar to that described previously (*9*). A single subcutaneous injection of each compound was given to CAF_1 mice bearing 6-day-old intramuscu-

TABLE 1.—*Melting points of the colchicine derivatives employed*

NCI No.	Compound	Melting point (° C.)
1987	Hexahydrocolchicine*	170 – 170. 5
2496	Hexahydrocolchiceine	204. 5 – 205. 5
2497	Colchinoic acid	262. 5 – 263
2498	Colchinoic acid methyl ester	253 – 253. 5
2628	N-Benzoylcolchinic anhydride	218. 5 – 219. 5
2778	Isocolchiceine ethyl ether	222 – 223
2798	Isocolchiceinamide	159 – 162
2824	Isocolchicine	222 – 223
2825	Desmethylcolchicine	{176 – 190; 275. 5 – 277
2826	Colchiceine ethyl ether	135 – 139
3126	Isotrimethylcolchicinic acid methyl ether *d*-tartrate·CH_3OH†	145 – 155 dec.

*Obtained from Professor D. S. Tarbell, University of Rochester. The melting point of this product is much higher than that of the specimen prepared by Bursian (*10*). Since reduction of the double bonds in the C ring increases the number of asymmetric carbon atoms, additional isomeric compounds are possible, as shown by Kemp and Tarbell (*11*) who obtained two different products, probably diastereoisomers, under different conditions of reduction. One was the product listed here; the other is probably identical with Bursian's compound.

†Loses methyl alcohol on drying *in vacuo*.

lar implants of Sarcoma 37. The animals, together with appropriate numbers of untreated tumor-bearing mice, were sacrificed 24 hours after the injection, and damage induced in the tumors was evaluated as described earlier. Only animals that survived were used in the evaluations; those that died were discarded.

Experimental Results

Table 2 summarizes the results obtained with the four *iso*-derivatives and with *colchiceine ethyl ether*. For ready comparison, the data for the other three normal analogs are reproduced from previous publications (*1*, *2*). Following a single injection of a small dose (2 or 3 μg./gm. of body weight), the normal forms all produced damage in Sarcoma 37, whereas huge doses (200 to 1,500 μg./gm.) of the *iso*-analogs failed to produce damage in the tumor. The lack of effect of the *iso*-compounds was not ascribable either to insolubility in water or to insufficient dosage, for the latter was pushed to the point where, with 3 of the 4 negative compounds, some mortality resulted.

TABLE 2.—*Damage induced in Sarcoma 37 with a single subcutaneous injection of derivatives of colchicine and isocolchicine*

NCI No.	Compound	Vehicle	Dose (μg./gm.)	Mortality*	Induced damage† after 24 hours
1v	Colchicine‡	water	2	0/7	7/7
2824	Isocolchicine	water	300	8/8	.....
			200	6/8	0/2
			150	0/8	0/8
2826	Colchiceine ethyl ether	water	5	0/8	8/8
			3	0/16	3/16
			2	0/8	0/8
2778	Isocolchiceine ethyl ether	olive oil	1,000	1/8	0/7
2177	Colchiceinamide‡	aqueous HCl	40	1/16	6/6
			2	0/15	3/15
2798	Isocolchiceinamide	olive oil	500	0/5	0/5
		aqueous HCl	400	0/5	0/5
1136	Trimethylcolchicinic acid methyl ether *d*-tartrate.‡	water	4	0/18	18/18
			2	0/20	8/20
3126	Isotrimethylcolchicinic acid methyl ether *d*-tartrate•CH_3OH.	water	1,500	4/5	0/1
			1,000	0/5	0/5
			750	0/5	0/5

*Expressed as: $\frac{\text{No. of mice dead}}{\text{No. of mice treated}}$.

†Expressed as: $\frac{\text{No. of mice with induced tumor damage}}{\text{No. of mice sacrificed}}$. The tumors in untreated controls showed no induced damage.

‡Data taken from earlier papers (*1*, *2*) for ready comparison.

Table 3 summarizes the findings with six other derivatives, only two of which showed potency in damaging Sarcoma 37. *Hexahydrocolchicine* was active at high doses. *Desmethylcolchicine* was active at about as low a dose level (5 μg./gm.) as colchicine and other highly active derivatives. Although unsaturation of the C ring was a feature of the structure of all the highly potent compounds, it obviously is not essential for potency, inasmuch as *hexahydrocolchicine* had some activity.

TABLE 3.—*Damage induced in Sarcoma 37 with a single subcutaneous injection of miscellaneous derivatives of colchicine*

NCI No.	Compound	Vehicle	Dose (μg./gm.)	Mortality*	Induced damage† after 24 hours
1987	Hexahydrocolchicine.....	water.....	665	0/5	5/5
			450	0/8	7/8
			300‡	0/16	1/16
2496	Hexahydrocolchiceine....	olive oil.....	1,000	0/8	0/8
2825	Desmethylcolchicine.....	water.....	20‡	0/16	8/8§
			10‡	0/16	8/8§
			5‡	0/16	12/16
			3	0/8	0/8
2497	Colchinoic acid.....	aqueous alkali...	1,000	0/8	0/8
2498	Colchinoic acid methyl ester.	olive oil.....	1,000	0/7	0/7
2628	N-Benzoylcolchinic anhydride.	olive oil.....	1,000	0/8	0/8

*Expressed as: $\frac{\text{No. of mice dead}}{\text{No. of mice treated}}$.

†Expressed as: $\frac{\text{No. of mice with induced tumor damage}}{\text{No. of mice sacrificed}}$. The tumors in untreated controls showed no induced damage.

‡Two separate experiments.

§Mice in one experiment were not sacrificed at 24 hours, but were observed for mortality over a period of approximately 5 days.

Table 4 gives approximate values for the maximum tolerated dose (MTD) and the minimum effective dose (MED) of those active compounds not previously reported by us. *Desmethylcolchicine* gave a ratio of

TABLE 4.—*Approximate values for the maximum tolerated dose (MTD) and minimum effective dose (MED) for a single subcutaneous injection of colchicine derivatives in mice bearing Sarcoma 37*

NCI No.	Compound	MTD * (μg./gm.)	MED * (μg./gm.)	MTD/MED
1987	Hexahydrocolchicine.....	>1,000	450	>2
2825	Desmethylcolchicine.....	40	5	8
2826	Colchiceine ethyl ether.....	8	3	3

* These values were determined in the fashion described in an earlier paper (9). Survival data for tumor-bearing animals that were not sacrificed for a period of about 5 days after injection, were used in estimating the values for MTD.

MTD/MED appreciably greater than that for colchicine (8 and 2, respectively). No deaths resulted with the highest dose of *hexahydrocolchicine* (1,000 μg./gm.).

Discussion

The results obtained with the *iso*-compounds emphasize the importance not only of the nature of the substituents, but also of their position in the C ring of the colchicine molecule. None of the four *iso*-compounds examined showed potency in damaging Sarcoma 37, whereas all the analogous compounds with the normal configuration were highly effective. Aqueous solubility did not appear to play the determining role, for three of the *iso*-compounds were sufficiently soluble in aqueous medium and the *isotrimethylcolchicinic acid methyl ether* d-*tartrate* appeared to be even more soluble than its normal analog. Moreover, at least two of the compounds with the normal structure [*colchicine* (*12*) and *colchiceinamide* (*2*)] proved to be active in arresting mitosis or in damaging Sarcoma 37 when injected as a suspension in oil. Furthermore, three of the four *iso*-compounds showed no potency in producing damage in the tumors even with doses at, or above, the maximum tolerated.

This absence of tumor-damaging activity of the *iso*-compounds is in accord with reports of low activity for other types of biological phenomena. Steinegger and Levan (*13*), in 1947, showed that *isocolchicine* had only about 0.01 the activity of *colchicine* in producing "c-mitosis" in the growing root tips of *Allium cepa*, and Šantavý and Reichstein (*4*) reported that *isocolchicine* and *isocolchiceine ethyl ether* were much less toxic for rats than their normal analogs.

Lettré and Fernholz (*14*) have recently reported that *isocolchicine* and *isocolchiceine ethyl ether* had only about 0.01 and 0.001, respectively, of the antimitotic activity of *colchicine* for fibroblasts *in vitro*. In the case of the Ehrlich ascites tumor, a dose of *isocolchicine* 10 to 20 times that effective in the case of *colchicine* failed to increase survival substantially. *Isocolchiceine ethyl ether* [6] also gave negative results, but the highest dose used was only about twice the minimum effective dose of the normal analog.

Alkylation of the hydroxyl group in the C ring of colchiceine yields two series of isomers which differ widely in their effects upon mitosis and upon tumors. Biological data obtained earlier, with reaction products containing mixtures of such isomers in unknown relative amounts (*16*), must be re-evaluated in the light of recent advances. Where physical data on the reaction product (*e.g.* melting point, optical rotation, etc.) were provided, some basis exists for reinterpretation of original conclusions. In the absence of such data, it is difficult to evaluate the significance of the biological data reported.

By means of physical data (infrared spectra and optical rotation), Horowitz and Ullyot (*17*) demonstrated the existence of hydrogen bond-

[6] The biological data given for this *iso*-derivative are identical with those reported earlier (*15*) for "krystallisiertes Äthylcolchicein." This crystalline product, for which no physical properties were reported in the earlier paper, was later (*14*) recognized as the *iso*-compound after publication of the report of Šantavý and Reichstein (*4*).

ing between the hydroxyl group of the C ring and the carbonyl group of the N-acetyl substituent on the B ring of colchiceine. They suggested that the greater part of *colchiceine* may be in the *iso*-form (text-fig. 2). Such an *iso*-structure is consistent with the much lower biological activity of *colchiceine* as contrasted with *colchicine*. Analogously, the *N-benzoyltrimethylcolchicinic acid*, which has low activity (*1*), may also exist mostly in the *iso*-form.

Colchiceinamide has higher activity (*2*) against Sarcoma 37 than *colchiceine*. In the former, the amino group is fixed in the normal position; there is no hydrogen bonding and therefore no possibility of production of the *iso*-form. In support of this point of view, *isocolchiceinamide*, prepared from *isocolchicine*, was found to be inactive at doses even 250 times greater than those with which positive results were obtained with the normal form, *colchiceinamide*.

In the past, investigators (*18–20*) who have concerned themselves with correlating structure and biological activity of colchicine derivatives have stressed the importance of the methoxyl group in the C ring of colchicine in imparting high biological activity to the compounds. In light of the observations with the *iso*-derivatives, the importance of the methoxyl group may be due to its fixing the configuration of these compounds in the normal form. The lower activity of the demethylated derivatives may be due to transformation, for the greater part, to the less potent *iso*-form, which is stabilized by hydrogen bonding. It is not possible at this time, however, to extend this hypothesis to all derivatives of *trimethylcolchicinic acid* of low potency. There is no evidence, for example, that *trimethylcolchicinic acid* itself, which damaged Sarcoma 37 only at doses of 600 μg. per gm. (*1*), exists largely in the *iso*-form. Its optical rotation is intermediate between that for the compounds of the *colchicine* and for the *isocolchicine* series.

Increasing the length of the alkyl chain on the hydroxyl group of the C ring (colchicine, in this connection, is considered the methyl ether of colchiceine) does not appear to increase the ratio MTD/MED. *Colchiceine ethyl ether* had virtually the same ratio as *colchicine*. Lettré and Fernholz (*19, 21*) [see summary table given by Ludford (*22*)], in fact, showed that potency decreased as the chain was lengthened from methyl to butyl.

Hydrogenation of the C ring to form *hexahydrocolchicine* (NCI-1987) did not abolish potency in our experiments. This compound caused no deaths at the highest dose given (1,000 μg./gm.), but required a large dose (450 μg./gm.) to produce damage in Sarcoma 37. Thus it would appear that unsaturation of the C ring is not essential for activity, although it obviously affected the level of potency. Brues and Cohen (*12*), and Lettré and Fernholz (*19*), on the other hand, obtained no biological activity with this compound except at lethal dose levels. Since these groups of investigators did not report the physical or chemical properties of the products they employed, it is not possible to decide whether they were identical with the product we used or with that prepared by Bursian

(see footnote, table 1). Since stereoisomerism in colchicine derivatives, or in compounds derived from podophyllin (*23*), has such a profound influence on tumor-damaging potency, apparent discrepancies between their findings and ours might be due to uncertainty as to the extent to which the preparations differed.

Since we had established (*1*) that degradation of the unsaturated seven-membered C ring to an aromatic six-membered ring yielded compounds with high potency, greater in some instances than that of their analogs with a seven-membered ring, it was somewhat unexpected to find that *colchinoic acid* (NCI-2497) was inactive in spite of its ready solubility in dilute alkali because of the carboxyl group. Lettré, Krapp, and Ochsenschlager (*24*) showed the presence of chromosome ring forms in cells of mouse ascites tumor following injection of 350 μg. of the *methyl ester* of *colchinoic acid* ("*allocolchicine*"). No information, however, was given on survival time of the tumor-bearing mice following treatment with this compound; thus it is not possible to assess the significance of the morphologic observation which they reported.

Desmethylcolchicine (NCI-2825) is the only derivative of colchicine used by us to date in which a substituent in the A ring was modified. The results suggest that all the methoxyl groups on this ring may not be essential for tumor-damaging potency. In fact, the ratio of MTD/MED for *desmethylcolchicine* was greater than for *colchicine* (8 *vs.* 2). This derivative is probably identical with "Compound C," which was isolated from the colchicum plant by Šantavý and Reichstein (*4*). Šantavý, Lang and Malinsky (*25*) investigated the effect of "Compound C" on mitosis in regenerating rat liver. In their experiments the ratio of the lethal dose of the *desmethyl*-compound to its minimum effective dose was about 5, compared with a ratio of about 8 for *colchicine*. Since the values of these ratios in our experiments with Sarcoma 37 were 8 and 2 for the *desmethyl*-compound and for *colchicine*, respectively, it would suggest a somewhat greater susceptibility of Sarcoma 37 tumor tissue to *desmethylcolchicine* than of regenerating rat liver tissue. In the experiments both of Šantavý *et al.* (*25*) and of our own, the lethality of the *desmethylcolchicine* was considerably less than that of *colchicine*.

Brun *et al.* (*26*) have also recently reported the effect of Compound C on mitosis using the "nipple test." No definite effects were reported. However the highest dose they employed was only 1 mg. per kg. or $\frac{1}{30}$ the lethal dose, and only $\frac{1}{4}$ the minimum effective dose reported for regenerating rat liver by Šantavý *et al.*, cited above. Similarly, they reported negative results with *isocolchicine*, but here too, the highest dose reported (1 mg./kg.) was only 2 to 4 times the MED for *colchicine* and only $\frac{1}{15}$ the lethal dose.

N-benzoylcolchinic anhydride (NCI-2628), known as Windaus' anhydride, has been of great academic interest to the organic chemist as one of the key compounds in the elucidation of the structure of colchicine. It is

the only derivative of colchicine in our series in which the C ring was degraded extensively. A dose of 1,000 μg. per gm. of this compound had no effect on Sarcoma 37. Structurally it is somewhat related to a small series of tropolones or benztropolones, containing a seven-membered ring with or without a fused benzene ring, which we have examined for tumor-damaging potency. Of this group of compounds, only one (*γ-thujaplicin*), isolated from western red cedar, showed activity in damaging Sarcoma 37; a near-lethal dose was required. The results obtained with these compounds will be reported in detail in a later paper.

It is evident from our results and from those obtained with some of these derivatives of colchicine by earlier investigators, that pronounced changes in their ability to damage or arrest tumor growth, or to arrest mitosis, are obtained by modifying the molecule. In several instances these changes have been accompanied by great reduction in lethality.

Little information is available on the extension of such findings to the clinical treatment of cancer. We are aware only of the report of Cramer and Broderson (*27*) on the local application of *N-ethylcolchiceinamide* in the treatment of cutaneous cancer in patients. Even in gout, for which colchicine has been extensively used, we did not find published data relating to use of these colchicine derivatives. Yet, prior to 1920, Fühner (*18*), Rüssemeyer (*28*) and Lipps (*29*) had investigated the toxicity and pharmacology of some of these derivatives from the point of view of their possible use in gout, and had shown that some of these compounds were tolerated in animals in much higher doses than colchicine. The higher activity and lower toxicity of this type of colchicine derivative have been discussed (*20*, *30*, *31*) in the light of possible extension to clinical investigation.

Summary

1) The *iso*-forms of four potent colchicine derivatives (*colchicine*, *colchiceine ethyl ether*, *colchiceinamide*, and *trimethylcolchicinic acid methyl ether* d-*tartrate*) were all inactive in damaging Sarcoma 37 following a single subcutaneous injection of a lethal, or near lethal, dose.

2) *Colchiceine ethyl ether* damaged Sarcoma 37 in a dose as low as 3 μg. per gm. The MTD for a single injection was about 8 μg. per gm., and the ratio MTD/MED was about 3.

3) *Desmethylcolchicine*, a compound isolated from U. S. P. colchicine, also damaged Sarcoma 37. The MTD for this compound was about 40 μg. per gm., the MED about 5 μg. per gm., and MTD/MED about 8.

4) *Hexahydrocolchicine*, a reduction product of colchicine, required a dose of 450 μg. per gm. for production of tumor damage.

5) *Hexahydrocolchiceine*, *colchinoic acid* and its *methyl ester*, and *N-benzoylcolchinic anhydride*, were all inactive against Sarcoma 37 at a dose level of 1,000 μg. per gm.

6) The relationship of the chemical structure and of isomerism of these colchicine derivatives to potency in damaging Sarcoma 37 is discussed.

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Cytological Studies of Tumors. IX. Characteristic Chromosome Individuality in Tumor Strain-Cells in Ascites Tumors of Rats [1,2]

SAJIRO MAKINO *and* KYOKO KANÔ,[3] *Zoological Institute, Hokkaido University, Sapporo, Japan*

Since malignant tumors represent forms of uncontrollable rapid growth occurring in an organism by a continuous series of descendent cell generations, their biological significance is very great. Recently, a new field of research on malignant tumors has been developed through the approaches of morphology, physiology, and biochemistry. The cytological investigation of tumors is especially important to the understanding of fundamental problems underlying phenomena of malignant growth. Cancerous cells possess a remarkable capacity to grow autonomously. Although many papers devoted to the cytology of cancerous tissue have already been published, much of the older work described merely high mitotic rates, remarkable frequency of mitotic abnormalities, and striking aberrations of chromosome numbers as universal characteristics of malignant tumors.

For several years, the authors have studied the cytology of the Yoshida ascites sarcoma and other similar tumors in rats (*5–8, 12*). A major conclusion to be drawn from these cytological researches is that the Yoshida sarcoma possesses a definite strain of tumor cells having its own characteristic chromosome constitution and maintaining malignancy as well as inherited capacity for autonomous growth. Such tumor strain-cells are characterized by a subdiploid complex of ± 40 chromosomes which constitute two distinct groups, one being represented by 22 to 24 rod-shaped chromosomes which probably derive unaltered from the original host tissue cell of the rat, and the other comprising 16 to 18 V- and J-shaped elements specific for tumor cells. Chromosomal individuality of tumor strain-cells remains unchanged through successive transplant generations from host to host, since they multiply by regular mitosis. The growth of the Yoshida tumor is primarily due to the proliferation of these strain-cells, a finding

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that is not paralleled in any previous study of tumors. This phenomenon is significant in connection with the mechanism of growth of malignant tumors, as well as with the question of how the tumor cells arose or were transformed from ordinary tissue cells. It was desirable, therefore, to obtain evidence on the possible occurrence of similar phenomena in other kinds of tumors.

Materials and Methods

Recently, two different kinds of transplantable ascites tumors in Wistar white rats, quite similar in nature to the Yoshida sarcoma, were established in our laboratory. They were induced by application of azo dyes and called the MTK-sarcomas I and II, respectively. In every respect—morphological features of tumor cells, rate of successive transplantations, life span of the tumor-bearing host, mitotic rate of the tumor cells in a transplant generation, types of mitotic abnormalities in tumor cells, variation of chromosome number, and behavior of tumor cells throughout a whole life span of the host—the MTK-sarcomas I and II closely resemble the Yoshida sarcoma [*vide* Tanaka and Kanô '51 (*12*)]. Another strain of the ascites tumor called the Hirosaki sarcoma has been kept in our laboratory; it is also a transplantable tumor in white rats, established by Dr. Usubuchi of the Hirosaki Medical College. It was said to originate from a spontaneous epithelial tumor developed on the neck of a white rat. The present paper records the results of some morphological investigations on the chromosomes of the tumor cells in these three different kinds of ascites tumors.

The study of the chromosomes was carried out by the use of smear preparations of the ascites tumor stained with aceto-carmine or acetic gentian violet. Details of the technique may be found in previous publications (*6*, *7*, *10*).

Observations

MTK-sarcoma I

Tanaka and Kanô (*12*) studied cytologically all phases of a transplant generation of MTK-sarcoma I. Particular attention was directed to the rate of mitosis, the variation of the chromosome number, and the behavior of tumor cells, in comparison with the Yoshida sarcoma. It was pointed out that, in addition to cells in abnormal mitosis, the MTK-sarcoma I consists of cells with well-balanced subdiploid chromosomes, about 40 in number, which multiply with almost regular mitotic behavior. Since cells having mitotic abnormalities or undergoing disintegration are unable to continue division much longer, the growth of the tumor was primarily attributed to the multiplication of the tumor cells showing regular mitosis with subdiploid chromosomes. These cells are characterized by a certain number of rod-, V- and J-shaped chromosomes of varying sizes. They have not changed in their chromosomal individuality through the transplant generations from host to host.

In the present study, the chromosomes of the MTK-sarcoma I strain-cells were morphologically analyzed in comparison to those of the Yoshida

sarcoma. In every case, the strain cells were characterized by a well-balanced subdiploid complex of chromosomes, fluctuating in number around 40. Further, the chromosome complex was found to be made up of two distinct groups, one comprising rod-shaped chromosomes and the other consisting of V- and J-shaped elements varying in size (figs. 1–4, 34–38). The chromosomes were arranged in the normal radial manner, the longer ones occupying peripheral positions surrounding the smaller ones. Nothing unusual was seen in the orientation of the chromosomes, nor in the spindle formation.

In a number of cells, the chromosomes were classified by comparing their characteristic shape and size and arranging them in a series of pairs.[4] Examples are given in figures 5 to 8. By closer investigation, it was determined that in the MTK-sarcoma I two types of tumor strain-cells occurred that differed in their chromosome complements. One of them was characterized by having from 23 to 25 rod-shaped chromosomes and from 16 to 17 V- and J-shaped ones (figs. 1, 2, 7, 8, 34, 35), whereas the other type possessed from 24 to 26 rods and 15 to 17 V's and J's (figs. 3, 4, 9, 10, 36–38). Interestingly, the former type of strain-cell possesses two outstanding large V-shaped elements, while the latter type of cell has only one such V-element. For convenience in description, these two types of strain-cells are designated as the 2V-type cell and 1V-type cell. The chromosome complement in the two types of cells can be formulated as follows:

2V-type cell: (23–25) R's + (16–17) V's & J's
1V-type cell: (24–26) R's + (15–17) V's & J's

In both the rod- and V- or J-shaped chromosome groups, there occurred some cases in which certain chromosomes were unpaired, or in which some elements existed in excess, probably due to non-disjunction, lagging or some similar abnormality.

The rod-shaped chromosomes present a rather evenly graded size-seriation, ranging from long rods to short rods. All of them, except a few very small ones, are of considerable size. In the group of V- and J-shaped chromosomes, some larger ones are clearly recognizable by their distinct configuration as V- or J-shaped bodies, while smaller ones are individually indistinguishable, all showing a constriction at the middle or near one extremity. Although they are not sharply bent at the constriction, the constant occurrence of the constrictions makes it highly probable that these chromosomes carry submedian or subterminal centromeres.

Certain evidence indicates a variation in the occurrence of the tumor cells of the two types. During the earlier generations of the successive transplantations the frequency in occurrence of the 2V-type cell was found to be higher than that of the 1V-type cell. In fact, during the period from the first to the 20th generation of transplantation, the proportion of the 2V-type cells to the 1V-type cells was approximately 3 : 1. In later generations of transplantation, the frequency of the 2V-type cells showed a gradual decrease. By the 50th generation, the proportion of the 2V-type cells to the 1V-type cells became about 1 : 3.5. Probably this is due to a relative

[4] Pairs may represent the two homologues, although it is quite uncertain whether the members of a pair are really homologous mates.

change of activity between the two types of tumor cells. No satisfactory cause for this change can be given at present. Possibly the decrease in number of the 2V-type cell is due to an absolute decrease in their activity. One might assume that in earlier generations of transplantations there is produced a particular condition of the ascites which favors active proliferation of the 2V-type cells. This may be, as it seems to the authors, caused by the production in the ascites of a certain substance supporting particularly the activity of these cells. In later generations, however, the production of this substance falls off in the ascites, and the 2V-type cells are unable to keep pace with the 1V-type cells which remain unaffected by the decrease in the activating substance. In this, as in the Yoshida sarcoma, it seems likely, furthermore, that the inner balance between nucleus and cytoplasm may be better conditioned in the 1V-type cell than in the 2V-type cell.

MTK-sarcoma II

Tanaka and Kanô (*12*) demonstrated in MTK-sarcoma II a strain of tumor cells which contributes primarily to the growth of the tumor by multiplying with a regular mitotic behavior. Such cells were found to be interspersed among cells showing abnormal mitosis. These cells were remarkable for their well-balanced subdiploid chromosomes, 40 or thereabouts in number, and they were normal in their metaphase configurations (figs. 5 and 6).

Morphological analysis of the chromosomes of these strain-cells revealed again that the chromosome complex consists of two distinct groups, one of which contains rod-shaped chromosomes and the other comprising V- and J-shaped elements. Compared with those of the MTK-sarcoma I, however, the tumor cells of this sarcoma are characterized by the presence of fewer V- and J-shaped chromosomes. Here, the number of the rod-shaped chromosomes ranges from 28 to 30, while V- and J-shaped elements number 11 to 13 (figs. 11 and 12). Thus, the chromosome formula of the tumor strain-cells of MTK-sarcoma II is represented by (28–30) R's+(11–13) V's & J's. As in the former case, the rod-shaped chromosomes show a continuous gradation in length. In the V- and J-shaped chromosome group, the presence of a solitary large V-shaped chromosome is very prominent, and other smaller ones are also clearly discernible by their distinct two-armed structure.

Hirosaki Sarcoma

It was stated by Dr. Usubuchi that the Hirosaki sarcoma developed originally from the transplantation of a spontaneous solid tumor of unknown origin found on the neck of a white rat. According to him, some crushed tissue pieces prepared from this solid tumor were inoculated in the peritoneal cavity of several white rats. One of them so treated produced in its peritoneal cavity a considerable amount of milky ascites which was a suspension of tumor cells. The rats receiving a transplantation of this tumor ascites in their abdominal cavities died after 10 or more days. By peritoneal transplantation of the tumor ascites, successive transplantations of this sarcoma have been continued from rat to rat. Though not completely analyzed, the essential nature of this sarcoma was said to be closely similar to that of the Yoshida sarcoma as well as the MTK-sarcomas.

In this Hirosaki sarcoma, as in others, various types of abnormal mitoses were observed throughout a transplant generation. The following mitotic abnormalities were noted: stickiness and coalescence of chromo-

somes, abnormal swelling or vacuolization of chromosomes, deformation of chromosomes into unusual bodies, disintegration of spiral chromonemata in the chromosomes, non-disjunction and lagging of chromosomes, appearance of chromosome bridges at anaphase and telophase, irregular chromosome distribution at anaphase, scattering or displacement of chromosomes at metaphase, extrusion of some chromosomes from the equatorial plate, abnormal orientation of chromosomes at metaphase, restitution nucleus, multinucleate cells and multipolar mitoses. It is clear that cells that are undergoing abnormal mitoses or are in process of disintegration cannot pursue active multiplication, and die sooner or later. Interspersed with cells showing mitotic abnormalities, groups of tumor cells which contained a well-balanced subdiploid complex of chromosomes and which occurred with high frequency were found. These cells multiply in a regular manner showing usual chromosome behavior. There is no doubt, considering the evidence observed in other ascites tumors, that these cells with well-balanced chromosomes are the tumor strain-cells which serve as primary contributors to growth of the tumor.

The chromosomes of these strain-cells were analyzed by comparing them to those of the Yoshida sarcoma and of both MTK-sarcomas. These strain cells possess a chromosome number of about 40, and are characterized by certain numbers of rod-, V- and J-shaped chromosomes of varying sizes (figs. 13–21). Closer examination revealed that there occurred at least five different types of cells, differing in their chromosome complex. In every cell type, the chromosome complement consists of two distinct groups, namely, the group containing rod-shaped chromosomes and that showing V- and J-shaped elements. Further, they are characterized by having without exception the prominent V-shaped chromosomes of large size, but these vary in number from one to five. Based on the number of large V-shaped chromosomes, the strain-cells were classified into the following five types: 1V-type cell [5] (fig. 21), 2V-type cell (figs. 13, 14), 3V-type cell (figs. 15, 16), 4V-type cell (figs. 17, 18, 39, 40) and 5V-type cell (figs. 19, 20, 41, 42). Each type of cell is characterized again by the different numbers of rod-, V- and J-shaped chromosomes (figs. 22–29). After a morphological analysis of the chromosomes in a number of cells, the chromosome constitutions of the five cell types were formulated as follows:

Cell type	Chromosome formula	Illustrated in figure
1V-type cell.....	(26–27) R's+(13–14) V's & J's.....	21
2V-type cell.....	(23–26) R's+(14–17) V's & J's.....	13–14, 22–23
3V-type cell.....	(20–24) R's+(16–19) V's & J's.....	15–16, 24–25
4V-type cell.....	(19–22) R's+(17–20) V's & J's.....	17–18, 26–27
5V-type cell.....	(18–21) R's+(17–19) V's & J's.....	19–21, 28–29

[5] The frequency of the 1V-type cell is very low (see table 1). It is doubtful whether they are of constant occurrence as a strain cell, or whether they result in consequence of non-disjunction of a V-element from the 2V-type cell. At present, however, they are tentatively treated as a separate type of strain cell.

The number of chromosomes observed in the five cell types is summarized in table 1. As seen in this table, the chromosomes vary in number from 36 to 42, so far as observed in this study. The most frequent chromosome numbers here observed were 40 for the 2V-type cell, 39 for both the 3V-type and 4V-type cells, and 38 for the 5V-type cell. It is interesting to see that the total number of chromosomes tends to decrease with increase in number of the large-sized V-chromosomes. For instance, the ranges of the most frequently occurring numbers of chromosomes were 39–41 in the 2V-type cell, 38–40 in the 3V-type cell, 37–40 in the 4V-type cell and 36–40 in the 5V-type cell, respectively. Closer observation made it clear that the number of large rod-shaped chromosomes tends to decrease with increase of large V-shaped chromosomes (figs. 22–29). This pattern, which involves an inverse numerical correlation between the large rod-shaped chromosomes and the large V-shaped ones, seems to permit an interpretation as to the origin of the large V-shaped chromosomes, though the specific causes for their origin remain unknown.

TABLE 1.—*Chromosome numbers observed in five types of tumor strain-cells of the Hirosaki sarcoma. (Observations in the 10th to 15th transplant generations.)*

Type of cells	Chromosome numbers							Number of cells observed
	36	37	38	39	40	41	42	
1V-type cell	.....	.....	1	.....	.....	.....	1	2
2V-type cell	.....	.....	2	5	7	6	.....	20
3V-type cell	.....	1	4	10	5	3	2	25
4V-type cell	.....	5	14	18	10	4	.....	51
5V-type cell	2	2	6	2	2	.....	.....	14

Observations were made upon the frequencies of the five cell types during comparable periods of a transplant generation. As shown in table 1, random observations indicated that about half of the 112 cells observed represented the 4V-type cell. Next, the frequency of the five cell types was studied in two different tumor-bearing rats based on daily preparations made through the 2nd to 7th day after transplantation of the tumor. The results are given in table 2, *a* and *b*. In the individual rat A (table 2 *a*), of a total of 140 cells observed, the frequencies obtained were 45.7%, 33.6%, 12.1%, and 8.6% for the 4V-type, 3V-type, 2V-type and 5V-type cells, respectively. In the individual rat B, as seen in table 2 *b*, the frequencies observed in a total of 67 cells were 49.2% for the 4V-type cell, 20.9% for the 3V-type cell, 17.9% for the 2V-type cell, 9.0% for the 5V-type cell, and 3.0% for the 1V-type cell. In short, the tumor cells of the 4V-type showed the highest frequency in both cases, and those of the 1V-type the lowest. This relationship might imply differences in the activity of the various types of tumor cells.

As in the MTK-sarcoma I, a difference of frequency in occurrence of the five cell types was also observed in the Hirosaki sarcoma in its later transplant generations. Namely, around the 40th generation of transplantation, the frequency of these strain cells showed a sudden change. The feature is clearly recognizable from data presented in table 3 which include the results of observations upon two individuals of the 43rd transplant generation. The results of these observations are remarkable in

TABLE 2.—*Daily frequencies of the tumor strain-cells of the Hirosaki sarcoma, as determined from metaphase cells. (Observations in the 14th transplant generation.)*

Days after transplantation: / Type of cells	2 days	3 days	4 days	5 days	6 days	7 days	Number of cells observed	Frequency
a) Individual A:								*Percent*
2V-type cell	4	4	1	3	1	4	17	12. 1
3V-type cell	9	8	9	7	9	5	47	33. 6
4V-type cell	6	12	12	9	13	12	64	45. 7
5V-type cell	1	1	3	1	2	4	12	8. 6
No. of cells	20	25	25	20	25	25	140	100. 0
b) Individual B:								
1V-type cell	0	0	0	2	0	0	2	3. 0
2V-type cell	1	3	3	3	1	1	12	17. 9
3V-type cell	2	3	2	1	4	2	14	20. 9
4V-type cell	8	4	5	7	5	4	33	49. 2
5V-type cell	1	0	0	3	1	1	6	9. 0
No. of cells	12	10	10	16	11	8	67	100. 0

showing a strikingly increased frequency of the 2V-type cells, constituting more than 80 percent of all cells observed, with a complete disappearance of the 4V-type and 5V-type cells, the former of which had occurred with highest frequency in the early generations. The explanation proposed for the similar case seen in the MTK-sarcoma I may also be applicable to the present case. In the Hirosaki sarcoma, the tumor cells of the 2V-type seem to excel in their metabolic activity, possibly due to a well-balanced intracellular condition allowing a more active proliferation than occurred in the other cell types.

TABLE 3.—*Daily frequencies of the tumor strain-cells of the Hirosaki sarcoma, as determined from metaphase cells. (Observations in the 43rd transplant generation.)*

Days after transplantation: / Type of cells	2 days	3 days	4 days	5 days	6 days	7 days	Number of cells observed	Frequency
a) Individual A:								*Percent*
1V-type cell	0	0	1	2	0	2	5	6. 9
2V-type cell	8	13	9	17	4	9	60	83. 3
3V-type cell	2	3	1	1	0	0	7	9. 7
b) Individual B:								
1V-type cell	1	0	3	1	2	0	7	8. 1
2V-type cell	9	12	9	21	15	8	74	85. 0
3V-type cell	0	0	1	1	2	2	6	6. 9
No. of cells	10	12	13	23	19	10	87	100. 0

Comparison of Chromosomes of Tumor Cells With Those of Ordinary Tissue Cells

A comparison of the chromosomes of the tumor strain-cells of the MTK and Hirosaki sarcomas with those of ordinary tissue cells of the host white rat discloses marked differences existing among them. The chromosomes of the Wistar white rats which were used for transmission of the tumor in this study were studied previously both in germ cells (*3*, *4*) and in somatic cells (*11*), establishing 42 diploid chromosomes in both cases. The chromosomes were, in both germ and tissue cells, of a simple rod type, tapering toward the inner end. No evidence of the presence of a V- or J-shaped chromosome has been found, so far as the observations have gone (figs. 30 and 31). In serial alignments of the paired chromosomes, the morphological differences between chromosomes from normal and from tumor cells are at once clear. A comparison of figures 30 and 31 with figures 7 to 10, 11, 12, and 22 to 29, reveals that, because of the apparent likeness in morphological features, the pairs of rod-shaped chromosomes occurring in tumor cells of each sarcoma seem to correspond to certain chromosome pairs in ordinary tissue cells. In other words, the rod-shaped chromosomes found in tumor cells probably are derived without change from the normal rat chromosomes. Since one cannot find any V- or J-shaped elements in ordinary tissue cells of rats, these chromosomes should be regarded as specific to the tumor cell.

As thus far noted, the chromosome constitutions of the tumor strain-cells studied in three kinds of tumor are clearly different from each other and specific according to the kind of tumor. Furthermore, the chromosomes of tumor cells in the tumors here studied are clearly distinguishable from those in the Yoshida sarcoma (*cf.* figs. 32 and 33 with figs. 7 to 10, figs. 11 and 12, and figs. 22 to 29). It was thus apparent that the tumor strain-cells of each of four kinds of ascites sarcomas possess their own characteristic chromosomes. The results of these studies are of significance in considering the origin of cancer cells.

Discussion

These investigations have made it clear that, in each of the four kinds of ascites tumors, three of which were experimentally induced and one of which was spontaneous in origin, there is present a strain (or strains) of tumor cells, having peculiarly characteristic chromosome constitution, that multiply with almost regular mitotic behavior. The individuality of chromosomes in these strain-cells has remained unchanged through successive transplant generations from host to host. The growth of the tumor is primarily the result of proliferation of these tumor strain-cells.

Earlier workers often assumed the variable chromosome numbers in tumor cells to be closely related to their malignancy. Indeed, malignant cells generally display a striking variability in chromosome number. For instance, the chromosomes of the Yoshida sarcoma vary in number over a range from about 20 to 80, and occasionally are found as high

polyploids (*8*). Also, the chromosome numbers in tumor cells of MTK-sarcoma I show a wide variation from about 26 to approximately 87 (*12*). However, it should be remembered that chromosome numbers likewise vary considerably in ordinary tissue cells. According to Tanaka (*11*), the tissue cells of various organs of young albino rats show a numerical variation of chromosomes from 36 to 84. A comparable condition has been reported by Therman and Timonen (*13*) in various human tissue cells. It is therefore quite reasonable to conclude that variation in chromosome number alone can hardly be taken as the ultimate cause of the malignancy of cancer. Apparently, many investigators of the cytology of tumors have directed their attention merely to the numerical variation of chromosomes, or to mitotic abnormalities occurring in tumor cells, and have overlooked changes in the constitution of the chromosomes.

In the present material, the implications are that changes more far-reaching than mere numerical change through mitotic abnormalities have occurred. The chromosome complexes of tumor cells in the four kinds of ascites tumors here considered show remarkable differentiation from those of the host. There is present a strain (or strains) of tumor cells having a specific chromosome constitution of constant individuality characteristic for each kind of tumor. A certain group of chromosomes in these tumor strain-cells is apparently of normal host origin because of the apparent likeness of the rod-type morphology. Another group consisting of V- and J-shaped elements appears to have arisen through transformation of normal tissue chromosomes, since they are entirely absent in the host cells. Thus, the condition seen in these sarcomas, in which a group of morphologically normal chromosomes is accompanied by a group of transformed chromosomes, indicates that tumor cells have arisen through alterations in the constitution of certain individual chromosomes of an ordinary tissue cell (or cells).

It has frequently been postulated without direct proof that the origin of the malignant cell, be it spontaneous or experimentally induced, must be essentially mutational. As an explanation for the origin of the particular chromosome complexes found in the tumor strain-cells of four kinds of ascites tumors, the occurrence of chromosome mutations during the transformation of an ordinary tissue cell (or cells) into a tumor cell (or cells) is most probable. Probably at some stage during experimentation with the carcinogenic agent, or in the life history of an individual, a mutational change took place in the chromosomes of an ordinary tissue cell (or cells). Perhaps, the process of mutation was such that it has not essentially disarranged the elements of cell metabolism or of mitotic activity, but enabled the mutated cells to grow autonomously. These cells multiplied to produce a strain (or strains) of tumor cells in which the altered chromosome constitution and the capacity for autonomous growth were preserved. The growth of the tumor in successive transplantations is primarily caused by continued proliferation of the tumor strain-cells. During the course of the proliferation, a number of the derivatives of these strain-cells became abnormal through aberrant mitoses, due probably to changes in the normal spindle mechanism, the structural alteration of chromosomes and some other unknown causes (*7*, *8*). But part of the strain-cells remained unchanged in their specific chromosome individuality, preserving not only their characteristic number of chromosomes but also their particular morphological constitution. Obviously, the cells showing mitotic abnormalities are unable to continue division much longer, and therefore they are of secondary importance for the

growth of the tumor. In this connection, Little (*2*) stated,". . . the cancer cell is not a diseased cell but rather one that performs its life cycle more rapidly and with an effectiveness at least equal to that of the normal cell. If then it is produced by the process of mutation, that process has not basically disarranged the elements of cell metabolism or of mitosis in the site in which the neoplasm occurs or to which it can metastasize." The phenomenon here stated by Little was partly demonstrated in tracing the mitotic course in the living tumor cell by Makino and Nakahara (*9*).

The occurrence of two types of tumor strain-cells with different chromosome complements in the MTK-sarcoma I and of five types of such cells in the Hirosaki sarcoma is of special interest in connection with transformation of tissue cells into malignant cells. These strain-cells possessed different chromosome complexes specific to each and, further, they differed in their mitotic activity. This fact implies that mutational change in the chromosome might have taken place independently in at least two different cells in the MTK-sarcoma I, and in five cells in the Hirosaki sarcoma. Also, these strain-cells not only showed different frequencies of occurrence by types of cells, but also a profound change of frequency between the early and later transplant generations (tables 2 and 3). Based on these findings, it is deduced that the mutational change in the chromosomes might occur not in a single but in several tissue cells in the original animal, producing several kinds of mutated cells differing in their chromosome constitutions. There might be produced inactive cells provided with poor intracellular balance. In the course of transplant generations such inactive mutated cells probably undergo degeneration, ultimately leaving a group of cells which are highly active in mitotic activity and in resistance due to a well-balanced nucleo-cytoplasmic relationship. The latter cells develop giving rise to a strain of tumor cells, the primary contributors to the formation of the tumor. This condition appears to characterize the Yoshida sarcoma and the MTK-sarcoma II, whereas the MTK-sarcoma I as well as the Hirosaki sarcoma are probably in a transitional state leading to this change.

The origin and nature of the two-armed chromosomes, V's and J's, occurring in the tumor strain-cells cannot be definitely explained at present, but some suggestive evidence has been found in the Hirosaki sarcoma. As already described, in the Hirosaki sarcoma there occur five different types of tumor strain-cells, each being characterized by one, two, three, four or five large V-shaped chromosomes. In these five types of cells, the number of large rod-shaped chromosomes decreases with the increase of large V-shaped elements. This inverse numerical correlation can be accounted for by the formation of the large V-shaped chromosomes through the fusion of two large rod-shaped elements at their kinetochore regions, an event of general occurrence in both animals and plants.

In discussing the origin of the malignant cell, Caspersson and Santesson (*1*) stated that the origin is to be sought in a change in the heterochromatic portions of chromosomes. This suggestion is based upon observations that nucleic acid metabolism is governed by the heterochromatic parts of the chromosomes, and that malignant cells contain more nucleic acid than normal tissue cells. In reference to the present authors' findings,

this view is interesting, because the observed change in shape of the chromosomes may bring about some internal alteration upsetting the normal relations between euchromatin and heterochromatin.

It must be realized, however, that the origin of the malignant cell cannot be attributed to a single cause; there are many types of malignant tumors differing in nature and in origin, and, accordingly, there should be several causes in the development of malignant tissues. We shall have to await the efforts of pathology, biology and chemistry to produce a more coherent and satisfactory picture before an explanation for the origin of malignant tumors may be found.

Summary

This paper deals with the chromosomes of tumor strain-cells in three different kinds of ascites tumors of albino rats, two experimentally induced and one of spontaneous origin. It was found that in each of the tumors studied, there is present a strain (or strains) of tumor cells having characteristic chromosome constitutions specific to the kind of tumor, and remaining unaltered in chromosome individuality through successive transplant generations. These strains of cells are primary contributors to the formation of the tumor. In every case, the tumor strain-cells show a well-balanced subdiploid complex of chromosomes, 40 or thereabouts in number. The chromosome complex consists of two distinct groups, namely a group of rod-shaped chromosomes and a group of V- and J-shaped ones. The three kinds of ascites tumors studied are characterized by different numbers of the rod- (R), V- and J-shaped elements. The chromosome formulae for the tumor strain-cells characteristic of the three kinds of the ascites tumors are represented as follows:

MTK-sarcoma I	1. 2V-type cell	: (23–25) R's+(16–17) V's & J's
	2. 1V-type cell	: (24–26) R's+(15–17) V's & J's
MTK-sarcoma II		: (28–30) R's+(11–13) V's & J's
Hirosaki sarcoma	1. 1V-type cell	: (26–27) R's+(13–14) V's & J's
	2. 2V-type cell	: (23–26) R's+(14–17) V's & J's
	3. 3V-type cell	: (20–24) R's+(16–19) V's & J's
	4. 4V-type cell	: (19–22) R's+(17–20) V's & J's
	5. 5V-type cell	: (18–21) R's+(17–19) V's & J's

The rod-shaped chromosomes found in tumor cells are probably unchanged normal chromosomes, while the V- and J-shaped ones have become transformed, since they are not to be found in normal nontumor cells. The exact origin and nature of these two-armed chromosomes remains unknown, but evidence from the Hirosaki sarcoma suggests that the large V-shaped chromosomes originated by fusion of two large rod-shaped chromosomes at their kinetochore regions.

As an explanation for the origin of the particular chromosome complexes occurring in the tumor cells, the occurrence of chromosomal mutations during the transformation of an ordinary cell or cells is most probable.

Addendum

After this paper was completed and ready to send to press, we found the paper by Hauschka and Levan entitled "Chromosome numbers of three mouse ascites tumors," Hereditas 38:251–255, 1952. It seems to us that the particular populations of tumor cells found in their material nearly correspond to the strain or stem-cells occurring in our material.

Further, one of the authors (Makino) has recently found, after the study of the *tissue culture* material, that *some* of the chromosomes in normal tissue cells of rats may not be simple rod-shaped ones, but of two-armed structure. Details may be described elsewhere after the completion of the study.

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PLATES

PLATE 132

Metaphase plates of tumor strain-cells. Camera-lucida. × *2,200*

FIGURE 1.—2V-type cell; 42 chromosomes. MTK-sarcoma I.

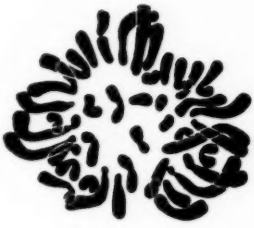
FIGURE 2.—2V-type cell; 40 chromosomes. MTK-sarcoma I.

FIGURE 3.—1V-type cell; 41 chromosomes. MTK-sarcoma I.

FIGURE 4.—1V-type cell; 42 chromosomes. MTK-sarcoma I.

FIGURE 5.—Metaphase plate of tumor strain-cell of the MTK-sarcoma II; 41 chromosomes.

FIGURE 6.—Metaphase plate of tumor strain-cell of the MTK-sarcoma II; 42 chromosomes.



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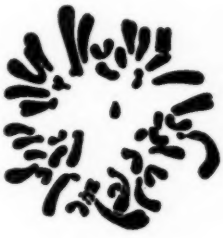

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PLATE 133

Serial alignments of paired chromosomes showing two groups of chromosomes in tumor strain-cells. Camera-lucida. × 2,200

FIGURE 7.—From 2V-type cells of MTK-sarcoma I.

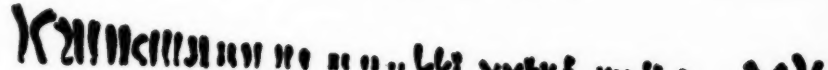
FIGURE 8.—From 2V-type cells of MTK-sarcoma I.

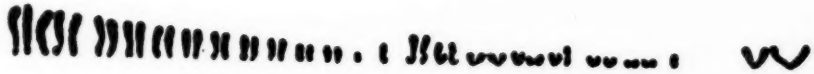
FIGURE 9.—From 1V-type cells of MTK-sarcoma I.

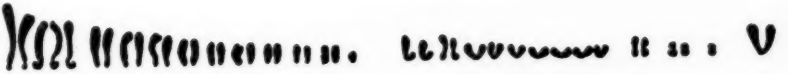
FIGURE 10.—From 1V-type cells of MTK-sarcoma I.

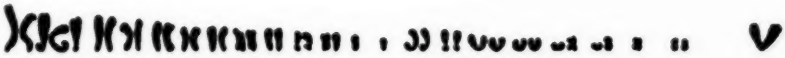
FIGURE 11.—MTK-sarcoma II.

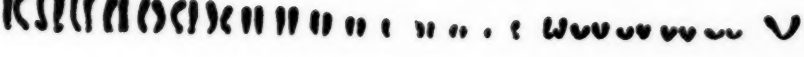
FIGURE 12.—MTK-sarcoma II.

7 

8 

9 

10 

11 

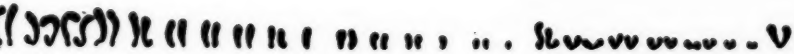
12 

PLATE 134

Metaphase plates of tumor strain-cells of the Hirosaki sarcoma. Camera-lucida. × 2,200

FIGURE 13.—2V-type cell; 38 chromosomes.

FIGURE 14.—2V-type cell; 40 chromosomes.

FIGURE 15.—3V-type cell; 40 chromosomes.

FIGURE 16.—3V-type cell; 39 chromosomes.

FIGURE 17.—4V-type cell; 39 chromosomes.

FIGURE 18.—4V-type cell; 39 chromosomes.

FIGURE 19.—5V-type cell; 38 chromosomes.

FIGURE 20.—5V-type cell; 36 chromosomes.

FIGURE 21.—1V-type cell; 40 chromosomes.

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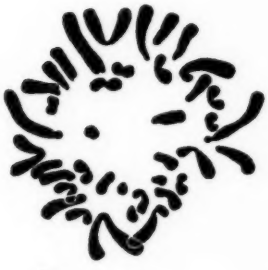

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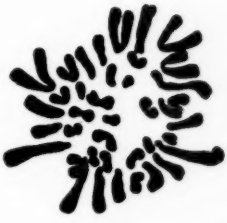

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PLATE 135

Serial alignments of paired chromosomes showing two groups of chromosomes in the Hirosaki sarcoma.

FIGURE 22.—From 2V-type cells.

FIGURE 23.—From 2V-type cells.

FIGURE 24.—From 3V-type cells.

FIGURE 25.—From 3V-type cells.

FIGURE 26.—From 4V-type cells.

FIGURE 27.—From 4V-type cells.

FIGURE 28.—From 5V-type cells.

FIGURE 29.—From 5V-type cells.

Serial alignments of supposed homologous pairs of chromosomes in tissues of Wistar albino rats.

FIGURE 30.—Blood cell.

FIGURE 31.—Hepatic cell.

Serial alignments of paired chromosomes showing two groups of chromosomes.

FIGURE 32.—Yoshida sarcoma cell.

FIGURE 33.—Yoshida sarcoma cell.

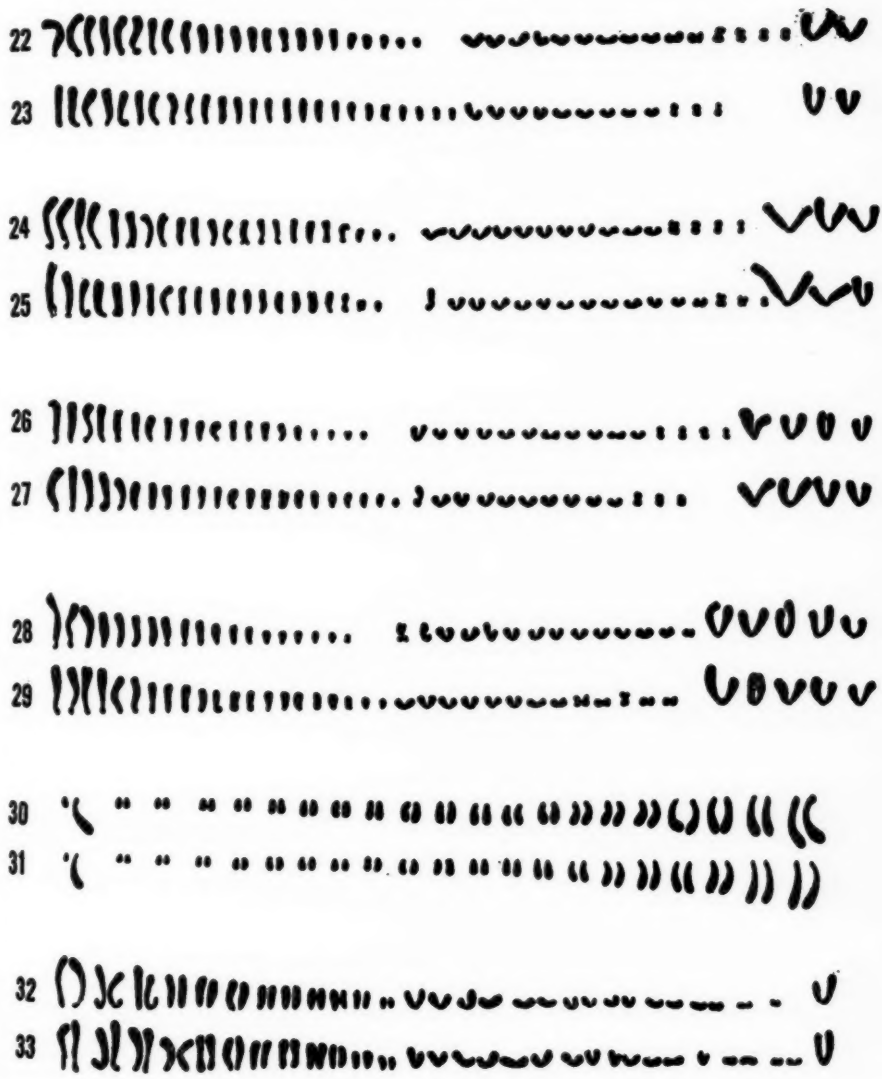

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PLATE 136

Photomicrographs showing metaphase plates of tumor strain-cells. × *1,200*

FIGURE 34.—2V-type cell of MTK-sarcoma I.

FIGURE 35.—2V-type cell of MTK-sarcoma I.

FIGURE 36.—1V-type cell of MTK-sarcoma I.

FIGURE 37.—1V-type cell of MTK-sarcoma I.

FIGURE 38.—1V-type cel lof MTK-sarcoma I.

FIGURE 39.—4V-type cell of Hirosaki sarcoma.

FIGURE 40.—4V-type cell of Hirosaki sarcoma.

FIGURE 41.—5V-type cell of Hirosaki sarcoma.

FIGURE 42.—5V-type cell of Hirosaki sarcoma.

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The Statistical Association Between Smoking and Carcinoma of the Lung [1]

DORIS A. SADOWSKY, ALEXANDER G. GILLIAM, *and* JEROME CORNFIELD, *National Cancer Institute,*[2] *Bethesda, Md.*

The question of the possible role of tobacco smoking in the etiology of cancer has stimulated several recent studies of the history of smoking among patients with cancer of various types. These investigations, conducted in several parts of the United States (*1–3*, *5*) and in England (*4*), have followed the familiar case-history pattern, where retrospective histories of smoking are obtained from patients with the illness under review and are then compared with similar histories of other persons not having that disease. They are generally consistent in showing a higher percentage of cigarette smokers among interviewed patients with lung cancer than found among other interviewed individuals with whom they were compared.

The present report records the results of a study of similar type that differs in two general respects from those previously recorded. First, the smoking habits of patients with cancer at several additional sites are presented, and second, the results are expressed in direct terms of the magnitude of the relative risk of cancer among smokers and nonsmokers. In addition, the findings are compared with those of several recently recorded investigations.

Source of Data

In most of the previous studies cited above, interviews of patients and controls were directed primarily toward ascertaining prior smoking history. In the present study a lengthy schedule was employed which provided retrospective histories on a variety of subjects, including smoking habits. These were collected by former personnel of the National Cancer Institute during the period 1938 to 1943, prior to the recent lay publicity regarding association between smoking and cancer. The general source of these records, as well as some consideration of their limitations, has been discussed previously (*6*). It should suffice to repeat here that the cases are believed to constitute a representative sample of patients coming for diagnosis and follow-up care to hospitals in New York City, Missouri, New Orleans, and Chicago. The controls

[1] Received for publication October 24, 1952.

[2] National Institutes of Health, Public Health Service, Federal Security Agency.

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TABLE 1.—*Total patients distributed by class of illness and type of smoking*

Type of smoking	Number of patients with cancer at specified site							Illness other than cancer	Total
	Lip	Tongue	Other oral cavity*	Pharynx	Esophagus	Larynx	Lung		
"Single" type of smoking:									
Cigarettes only	249	55	124	53	62	164	273	328	1, 308
Cigars only	13	8	23	2	5	6	11	21	89
Pipes only	104	23	67	8	8	13	13	43	279
Total "single" type	366	86	214	63	75	183	297	392	1, 676
"Mixed" type of smoking:									
Cigarettes and cigars	22	6	13	2	2	11	37	25	118
Cigarettes and pipes	81	19	33	6	12	44	78	62	335
Cigars and pipes	34	11	45	1	6	9	14	24	144
Cigarettes, cigars, and pipes	22	3	18	4	5	15	33	31	131
Total "mixed" type	159	39	109	13	25	79	162	142	728
Total smokers	525	125	323	76	100	262	459	534	2, 404
Nonsmokers	46	7	25	9	4	11	18	81	201
Total patients	571	132	348	85	104	273	477	615	‡2, 605
Percent who smoked	91. 9	94. 7	92. 8	89. 4	96. 2	96. 0	96. 2	86. 8	92. 3
Percent who smoked cigarettes†	65. 5	62. 9	54. 0	76. 5	77. 9	85. 7	88. 3	72. 5	72. 6

*Comprises cancer of the floor and roof of mouth, gum, buccal mucosa, palate, and tonsil.
†Those who smoked cigarettes only, or cigarettes together with cigars and/or pipes.
‡Records for 242 additional interviewed patients were excluded because of incomplete or questionable smoking histories.

were persons coming to the same hospitals with illnesses other than cancer. Though the cases are undoubtedly not truly representative of all cases of cancer of these sites occurring in the respective communities, most other selective factors involved are probably comparable in cases and controls. Since all interviews were conducted by four especially trained lay interviewers, personal bias in record-taking should have been reduced to a minimum.

The 2,605 records analyzed in this report were taken from a total of 2,847 records secured from white male patients with illnesses other than cancer or with primary cancer at one of the following sites: lip (upper and lower), tongue, other oral cavity,[3] pharynx, esophagus, larynx, and lung. The number for each class of illness, together with broad classification of their smoking history, is listed in table 1. The 242 records for other white male patients with these illnesses, which

[3] Comprises floor and roof of mouth, gum, buccal mucosa, palate, and tonsil.

have not been utilized, were excluded because of incomplete or questionable smoking histories.[4]

Classification of Smoking Habits

With human cancers that are believed to be precipitated by exposure to chemical carcinogens, degree and duration of exposure are generally considered to be directly related to tumor production. It is, therefore, not unreasonable to expect that these factors may be important if smoking is etiologically related to cancer.

It is general experience that smoking habits do not remain fixed throughout life. The habit starts at some age after which the quantities and types of tobacco smoked may vary considerably from time to time, and frequently may be interspersed with periods of partial or complete abstinence. It further seems clear that retrospective histories of smoking are not likely to give a very precise account of actual past smoking habits. However, unless cancers of some types independently tend to select individuals with more accurate memories, there seems little reason to believe that this factor operates differently in the persons with different classes of illness under study here. Any serious bias on the part of the interviewer appears unlikely in this study since smoking history was only one of a larger number of habits, etc., covered. Even, however, if accurate records kept during a lifetime were available, the bookkeeping involved in summarizing smoking histories would introduce difficulties.

For the purposes of this study some of these classification difficulties are arbitrarily solved. It is seen in table 1 that of the 2,404 patients who smoked, 1,676 (70 percent) gave "pure" smoking histories; that is, they claimed to have smoked only cigarettes, pipes, or cigars. The remainder (728 or 30 percent) smoked a combination of two or more of these types of tobacco ("mixed" smoking). The 201 patients who are classed as nonsmokers smoked "very rarely," "very occasionally," or not at all. In tabulating quantity of smoking per day for "mixed" smoking, cigars and pipe tobacco are converted to their tobacco equivalents in cigarettes as indicated in table 2.

The quantities smoked, which are tabulated, represent the record given for the patient's earliest smoking habits. This amount is thus assumed to exist throughout the time the patient smoked. This period was selected, rather than the period for the maximum ever smoked, or the period of most recent smoking, since it represents the interval farthest removed from development of the illness in question. It is assumed, in conformity with experience with carcinogens in experimental animals, that if smoking is cancer-producing, that indulged in just prior to disease is less important than that indulged in at a more remote time.[5]

[4] In many of the 242 excluded records, history was available for one or two types of smoking but was not recorded for another. A trial tabulation was made of the lung cancer cases including those for which such partial smoking history was recorded. The difference in results from those given in subsequent tables was negligible and these 242 cases were entirely excluded from all computations presented here.

[5] Tabulations for the lung cancer and control patients were also made for two assumptions, that *a*) the maximum ever smoked, and *b*) the quantity most recently smoked, was the quantity used throughout. No material differences in general findings were made by these procedures.

TABLE 2.—*Classification of quantity of tobacco smoked*

Cigarettes	Cigars	Pipes*	Mixed†
None‡	None ‡	None ‡	None.‡
10 or less	1 or less	0–¼ can	10 or less.
11–20	2	¼–½ can	11–20.
21–30	3	½ can or more	21–30.
31–40	4		31–40.
41 or more	5 or more		41 or more.

*One can of pipe tobacco is equal to two ounces of tobacco.

† Mixed smoking is defined as non-"pure" smoking (smoking of cigarettes and cigars; cigarettes and pipes; cigars and pipes; or cigarettes, cigars, and pipes). According to information obtained by personal communication from Mr. Claude Turner of the Department of Agriculture, Bureau of Agricultural Economics, Tobacco Branch, there are approximately 30 pounds of tobacco per 1,000 average cigars and 3.12 pounds of tobacco per 1,000 cigarettes on the "Farm Sales Weight" basis. On the basis of this "by weight" information, 1 cigar was used as the equivalent of 10 cigarettes, 0–¼ can of pipe tobacco as the equivalent of 10 or less cigarettes, ¼–½ can of pipe tobacco as the equivalent of 11–20 cigarettes, and ½ can or more of pipe tobacco as the equivalent of 21 or more cigarettes. Thus, if a patient smoked 12 cigarettes per day and 1 cigar per day (the equivalent of 22 cigarettes per day) he was classified in the class (of "mixed" smokers) 21–30.

‡ Nonsmokers are defined as patients who smoked "very rarely," "very occasionally," or not at all.

For each class of illness and type of smoking, the average ages at diagnosis and at onset of smoking, and the average duration of smoking, were computed. These data are not presented since no differences of apparent significance were developed.

The average ages at diagnosis were also calculated for each class of illness according to quantities smoked. If smoking were etiologically related to cancer it might be expected *a priori* that those smoking the largest amounts would develop illness at a younger age than those smoking smaller amounts. These data are not shown, but no regular relationship was discernible between average age at diagnosis and quantities of tobacco of various types smoked.

Method of Computation of Prevalence Rates

The method generally employed in demonstrating an association between tobacco smoking and cancer has been to compare a supposedly representative group of cancer patients with another group, selected as controls, on the basis of the percentage of smokers and nonsmokers in each group. While this procedure may demonstrate significant differences in the percentages of smokers in the groups compared, it provides no estimate of the magnitude of the relative risk of cancer among those who smoke and those who do not.

The procedure utilized in this study estimates relative risk of cancer among smokers and nonsmokers on the basis of prevalence rates computed by a method due to Cornfield (7). The two assumptions involved in computing these rates are also implicit in any comparison of a group of cases with control individuals such as those made in the previous studies mentioned. These assumptions are *a*) that insofar as prior history of smoking is concerned, the patients interviewed comprised a representative sample

of all cases of cancer at the particular site and *b*) that the controls interviewed similarly comprised a representative sample of individuals escaping cancer at that site. Estimation of the magnitude of the relative prevalence of cancer among smokers and nonsmokers requires, additionally, a knowledge of the approximate prevalence of that cancer in the population from which cases and controls were drawn.

Cornfield's formula (*7*) for computing prevalence rates of cancer among smokers and nonsmokers is duplicated as table 3. This utilizes for each age group, in each cancer site, the percentage of smokers and nonsmokers in patients with cancer and in control individuals, and the age-specific prevalence rate for cancer at that site in the general population. The latter rates utilized in this study are shown in table 4 and were calculated from rates of prevalence found in 1937 in surveyed areas of the United States as published by Dorn (*8*).

TABLE 3.—*Formula* for estimating prevalence rates of cancer among smokers and nonsmokers in each age group*

Attribute	With cancer	Without cancer	Total
Smokers	P_1X	$P_2(1-X)$	$P_2+X(P_1-P_2)$
Nonsmokers	$(1-P_1)X$	$(1-P_2)(1-X)$	$(1-P_2)+X(P_2-P_1)$
Total	X	$1-X$	1

*Method proposed by Cornfield (*7*).

Where P_1=the proportion of smokers among those with cancer.
P_2=the proportion of smokers among those with illness other than cancer.
$1-P_1$=the proportion of nonsmokers among those with cancer.
$1-P_2$=the proportion of nonsmokers among those with illness other than cancer.
X=the proportion of the general population in specified age group which has cancer of the particular site. The values of X utilized—site and age-specific prevalence rates—are given in table 4.

and r=prevalence rate $\begin{cases} =\text{for smokers}=[P_1X]/[P_2+X(P_1-P_2)] \\ =\text{for nonsmokers}=[(1-P_1)X]/[(1-P_2)+X(P_2-P_1)]. \end{cases}$

TABLE 4.—*Prevalence rates of cancer at several sites. Proportion per 100,000* general United States white male population which had cancer at a particular time (1937).*

Age	Site of cancer						
	Lip	Tongue	Other oral cavity	Pharynx	Esophagus	Larynx	Lung
Under 50	2. 67	1. 47	2. 40	. 47	1. 52	2. 21	3. 49
50–59	27. 24	15. 02	24. 52	4. 79	19. 13	25. 58	40. 38
60–69	64. 92	35. 78	58. 45	11. 42	45. 55	38. 48	60. 75
70 and over	106. 60	58. 76	95. 97	18. 75	70. 10	36. 76	58. 04
All ages	12. 15	6. 70	10. 94	2. 14	8. 09	8. 08	12. 76

*These rates were computed from Dorn's (*8*) data [table 7 (page 32) and Appendix table 3 (page 41)]. They are thus the approximate age-specific prevalence rates for surveyed areas in the United States in 1937.

TABLE 5.—*Prevalence rates of cancer per 100,000 white males by site of cancer, age at diagnosis, and type of smoking*

Site of cancer and type of smoking	Age at diagnosis					
	Under 50	50–59	60–69	70 and over	All ages	
					Crude	Standardized †
Lip:						
Cigarettes only	2.5	29.2	33.7	21.9	9.9	8.2
Cigars only	1.6	13.0	76.0	32.8	8.1	9.0
Pipes only	4.0	61.8	149.4	135.3	*31.7	25.0
Mixed	3.8	26.1	67.5	155.8	*14.7	16.4
Total smokers	2.8	29.3	70.0	112.8	*12.9	14.4
Nonsmokers	1.5	13.2	37.3	82.0	7.4	8.3
Tongue:						
Cigarettes only	1.4	16.8	19.0	23.0	5.2	5.0
Cigars only	0.0	19.9	87.5	138.2	*11.9	13.4
Pipes only	3.7	24.2	63.7	60.5	*16.7	12.1
Mixed	1.6	15.2	41.9	121.0	*8.6	10.4
Total smokers	1.5	16.9	39.4	73.4	7.3	8.5
Nonsmokers	1.2	2.5	15.9	0.0	2.7	2.3
Other oral cavity:						
Cigarettes only	1.8	23.7	35.7	29.8	7.3	7.5
Cigars only	8.0	22.2	80.3	267.8	*21.1	24.6
Pipes only	4.3	50.4	109.7	117.2	*30.1	20.6
Mixed	4.0	27.9	62.9	122.7	*14.8	15.1
Total smokers	2.6	26.5	61.9	111.6	*11.7	13.4
Nonsmokers	1.2	11.0	39.5	33.5	6.0	6.1
Pharynx:						
Cigarettes only	0.5	5.9	13.2	125.0	2.5	6.8
Cigars only	0.0	0.0	33.7	0.0	1.5	2.3
Pipes only	0.3	9.7	18.4	0.0	2.9	2.5
Mixed	0.2	4.4	6.6	0.0	1.4	1.1
Total smokers	0.4	5.3	12.7	23.4	2.2	2.6
Nonsmokers	0.7	1.3	4.6	0.0	1.7	1.0
Esophagus:						
Cigarettes only	2.1	23.0	55.3	200.3	*9.0	15.7
Cigars only	2.5	0.0	115.3	200.3	11.4	17.6
Pipes only	0.0	17.2	42.0	50.1	8.9	6.6
Mixed	0.9	23.3	35.2	50.1	8.4	7.5
Total smokers	1.7	21.5	48.1	87.6	*9.0	10.3
Nonsmokers	0.0	3.5	31.5	0.0	2.4	2.5
Larynx:						
Cigarettes only	2.6	31.8	56.6	0.0	*9.1	9.2
Cigars only	0.0	12.0	0.0	169.7	5.2	8.0
Pipes only	0.0	14.6	23.6	35.3	5.5	4.5
Mixed	2.7	26.7	48.1	70.7	*10.1	10.9
Total smokers	2.4	28.1	44.4	46.0	*8.9	9.6
Nonsmokers	0.8	8.9	5.9	0.0	2.5	2.0
Lungs:						
Cigarettes only	3.5	44.9	96.3	110.5	*13.7	18.3
Cigars only	3.3	17.0	19.0	165.9	8.6	12.2
Pipes only	1.8	11.6	20.6	20.7	5.0	4.8
Mixed	5.3	55.1	76.5	82.9	*18.8	18.3
Total smokers	3.8	44.5	70.9	62.2	*14.1	14.9
Nonsmokers	1.0	12.6	5.2	41.5	3.7	4.1

*Prevalence rates among smokers significantly greater than those among nonsmokers at a probability level of $P = .01$ or less.
†Standarized for age using white male population of the United States, 1940.

Estimated Prevalence of Cancer Among Smokers and Nonsmokers

Estimates of cancer prevalence rates among smokers and nonsmokers have been computed by the method outlined above. In table 5 these rates are given for each class of illness, by age of patient at diagnosis, and by type of smoking indulged in. It is observed that though alterations of rates result from age standardization, these differences do not materially affect the broad associations developed. For this reason subsequent consideration will be limited to crude rates, except in the instance of certain comparisons of results in this study with those of other workers.

In table 6 estimated crude prevalence rates are given for each class of illness and for each type of smoking—those smoking only cigarettes, cigars or pipes and those smoking some combination of them. In table 7 similar rates are presented for each site of cancer according to the quantities of tobacco smoked per day. For convenience of discussion, pertinent data from these three tables will be considered for each type of cancer separately.

TABLE 6.—*Estimated crude prevalence rates of cancer per 100,000 white males by type of smoking*

Type of smoking	Site of cancer						
	Lip	Tongue	Other oral cavity	Pharynx	Esophagus	Larynx	Lung
"Single" type of smoking:							
Cigarettes only	9.9	5.2	7.3	2.5	*9.0	*9.1	*13.7
Cigars only	8.1	*11.9	*21.1	1.5	11.4	5.2	8.6
Pipes only	*31.7	*16.7	*30.1	2.9	8.9	5.5	5.0
"Mixed" type of smoking:							
Cigarettes and cigars	4.6	7.5	10.1	1.2	3.8	8.0	*24.3
Cigarettes and pipes	*42.4	*9.6	10.3	1.5	9.3	*12.9	*20.7
Cigars and pipes	*18.6	*14.3	*36.3	0.6	12.0	6.8	9.6
Cigarettes, cigars, and pipes	9.3	3.0	11.2	2.0	7.7	*8.8	*17.5
Total "mixed" type	*14.7	*8.6	*14.8	1.4	8.4	*10.1	*18.8
Total for smokers	*12.9	7.3	*11.7	2.2	*9.0	*8.9	*14.1
Total for nonsmokers	7.4	2.7	6.0	1.7	2.4	2.5	3.7
Total for United States population (including smokers and nonsmokers)	12.2	6.7	10.9	2.1	8.1	8.1	12.8

*Indicates rate is significantly greater than that estimated for nonsmokers at a probability level of $P=.01$ or less.

Note: For the number of cases on which each rate is computed, see table 1.

TABLE 7.—*Estimated crude prevalence rates of cancer per 100,000 white males by site, type of smoking, and quantity smoked*

Quantities of tobacco smoked per day	Site of cancer						
	Lip	Tongue	Other oral cavity	Pharynx	Esophagus	Larynx	Lung
Cigarettes only:							
10 or less	10.5	2.4	6.3	3.6	8.7	7.1	9.0
11–20	9.6	6.4	7.6	2.2	8.4	9.0	13.2
21–30	10.1	4.0	8.7	2.0	10.8	6.5	15.9
31–40	11.6	5.5	3.4	1.8	16.9	13.9	30.1
41 or more	8.7	10.4	19.2	0.0	0.0	66.5	49.2
All quantities	9.9	5.2	7.3	2.5	9.0	9.1	13.7
Cigars only:							
1 or less	8.3	0.0	7.0	0.0	4.3	0.0	6.0
2	26.6	0.0	58.8	0.0	0.0	36.9	16.7
3	12.9	30.9	38.1	0.0	23.5	9.1	16.2
4 or more	3.7	26.7	33.1	4.4	20.4	7.8	9.4
All quantities	8.1	11.9	21.1	1.5	11.4	5.2	8.6
Pipes only:							
0–¼ can	22.5	13.9	17.2	2.6	7.9	3.0	4.6
¼–½ can	35.1	9.9	37.6	2.4	10.1	6.7	5.2
½ can or more	47.7	46.6	44.9	5.1	7.9	9.1	5.5
All quantities	31.7	16.7	30.1	2.9	8.9	5.5	5.0
Mixed:*							
20 or less	11.8	4.7	7.7	0.8	9.6	9.1	15.6
21–30	19.3	6.6	12.2	2.4	5.0	8.2	20.3
31–40	13.1	7.8	12.7	1.0	9.0	14.2	17.0
41 or more	13.3	12.0	20.8	1.2	10.1	9.5	19.9
All quantities	14.7	8.6	14.8	1.4	8.4	10.1	18.8
Total smokers	12.9	7.3	11.7	2.2	9.0	8.9	14.1
Nonsmokers	7.4	2.7	6.0	1.7	2.4	2.5	3.7

*Pipe tobacco and cigars are converted to their tobacco equivalent of cigarettes as indicated in table 2.

Cancer of the lip.—Estimated rates significantly [6] higher than in nonsmokers are found (table 6) for those smoking pipes only, and for those smoking cigarettes and pipes or cigars and pipes. In these smoking classes the ratios of the rates for smokers to nonsmokers vary from 2.5 to 5.7. The rates for those with other smoking habits, including those smoking a combination of cigarettes, cigars, and pipes are not significantly excessive.

In table 5 it is noted that excessive rates are found in every age class for pipe and "mixed" smokers, but that no such regular relationship obtains for other types of smoking. It is further observed in table 7 that a regular relationship between quantities smoked and increased rates is only observed among pipe and "mixed" smokers.

These facts, taken together, warrant the assumption that the association between pipe smoking and cancer of the lip is probably real in this series of patients.

[6] A rate among smokers higher than that observed among nonsmokers, at a probability level of $P=.01$ or less is regarded throughout as statistically significant.

Cancer of the tongue.—In this class of patients excessive rates were estimated (table 6) for those smoking either pipes or cigars; for those smoking pipes and cigars; and for those smoking cigarettes and pipes. The ratios of the rates among such smokers to nonsmokers varied from 3.5 to 6.2. When age-specific rates are considered (table 5) the rates are regularly excessive in all classes of smokers. With regard, however, to quantities smoked (table 7), rates increase regularly with quantity only among the "mixed" smokers.

Because of these inconsistencies and the relatively small numbers of cases represented for each subdivision of smoking history, some doubt must remain as to whether or not the association apparent between pipe and cigar smoking and cancer of the tongue is real in patients of this series.

Other oral cavity cancer.—For cancer of the oral cavity—exclusive of cancer of the tongue—significantly excessive rates are estimated (table 6) for those smoking only cigars or pipes and for those smoking cigars and pipes combined. The ratio of these rates to that for nonsmokers varies from 3.5 to 6.0 When age-specific rates are considered (table 5) they are regularly excessive for the same type of smokers. The relationship between quantity smoked and rates (table 7) is regular, however, only for the "mixed" and pipe smokers.

It may therefore be concluded that an association between pipe and possibly cigar smoking and cancer of the oral cavity is apparent in this series of cases.

Cancer of the pharynx.—Reference to tables 5, 6, and 7 will show that there is no suggestion whatsoever of any association between any type of smoking and pharyngeal cancer in this series of cases. The number of cases available for review is small, however.

Cancer of the esophagus.—Significantly excessive rates are estimated (table 6) for those smoking cigarettes only and for all smokers. The rates for those smoking other types of tobacco, including those with cigarettes in various combinations, are greater than in nonsmokers, but not significantly so. There is similarly no regular relationship shown (table 7) between quantities smoked and increase in rates. Thus, though an association between cigarette smoking and cancer of the esophagus is suggested, it cannot be regarded as demonstrated in this small series of cases.

Cancer of the larynx.—It is observed in table 6 that significantly excessive laryngeal cancer rates are estimated for those smoking cigarettes only and for those smoking cigarettes and pipes combined, and cigarettes, cigars, and pipes in combination. The ratios of rates among such smokers to nonsmokers vary from 3.5 to 5.2. With regard to age-specific rates (table 5) a regular relationship is shown, but it also exists for other classes of smokers. Estimated rates are also seen (table 7) to increase with intensity of smoking for all types of smoking, except cigars alone.

These data considered together, apparently warrant the conclusion that there is an association between cigarette smoking and laryngeal cancer in this series of cases.

Cancer of the lung.—For cancer of the lung significantly excessive rates are estimated (table 6) for persons smoking cigarettes only and for all types of smoking in which cigarettes appear. The ratios of the rates estimated for smokers and nonsmokers vary between 3.7 and 6.6. A regular relationship is also observed (table 5) in age-specific rates. Similarly, (table 7) rates increase with intensity of smoking for cigarette smokers and for pipe smokers but not for the other types.

These observations therefore justify the conclusion that in this series of cases there is an association between cigarette smoking and cancer of the lung.

Influence of Duration of Smoking Upon Estimated Prevalence Rates

Other authors have emphasized the fact that a high proportion of patients with cancer of the lung have smoked for 20 years or more. Because of the emphasis attached to this fact, rates have been estimated for cancer at each site, for those who smoked either cigarettes, cigars, or pipes ("single" type of smoking), according to the duration of smoking prior to diagnosis. These data are shown in Appendix Table A. Similar

Table 8.—*Estimated crude prevalence rates of cancer of the lung per 100,000 white males; distributed by duration and type of smoking and by age at diagnosis*

Age at diagnosis	Duration of smoking in years					
	Less than 20	20–29	30–39	40 and over	All durations	Non-smokers
	Cigarettes only					
Less than 50	1.5 (8)*	3.6 (59)	4.2 (50)	7.8 (4)	3.5 (121)	1.0 (7)
50–59	6.0 (1)	34.7 (9)	43.1 (67)	71.5 (32)	44.9 (109)	12.6 (8)
60 and over	—† (1)	20.2 (1)	—† (3)	99.8 (38)	99.9 (43)	12.1 (3)
All ages	4.3 (10)	10.3 (69)	14.6 (120)	27.1 (74)	13.7 (273)	3.7 (18)
	Cigarettes and other types					
Less than 50	5.1 (7)	4.4 (19)	11.2 (25)	0.0 (0)	6.3 (51)	1.0 (7)
50–59	41.2 (1)	39.2 (12)	73.0 (43)	54.0 (14)	59.4 (70)	12.6 (8)
60 and over	0.0 (0)	10.6 (2)	—† (2)	86.9 (23)	77.7 (27)	12.1 (3)
All ages	10.9 (8)	13.6 (33)	30.3 (70)	21.7 (37)	20.6 (148)	3.7 (18)
	Cigars and/or pipes (no cigarettes)					
Less than 50	1.7 (2)	1.9 (5)	2.0 (3)	?‡ (0)	1.9 (10)	1.0 (7)
50–59	3.8 (1)	16.9 (2)	37.8 (8)	0.0 (0)	16.6 (11)	12.6 (8)
60 and over	15.1 (1)	30.4 (1)	50.3 (5)	35.5 (10)	35.4 (17)	12.1 (3)
All ages	3.0 (4)	6.0 (8)	11.0 (16)	8.2 (10)	7.1 (38)	3.7 (18)

*Numbers in parentheses indicate number of lung cancer cases on which rate is based.
†No case in controls.
‡No lung cancer and no control case. Where rate in table is 0.0 there was no lung-cancer case.

TABLE 9.—*Estimated crude prevalence rates of cancer of the lip per 100,000 white males; distributed by duration and type of smoking and by age at diagnosis*

Age at diagnosis	Duration of smoking in years					
	Less than 20	20–29	30–39	40 and over	All durations	Non-smokers
	Pipes only					
Less than 50.	2.4 (2)*	2.7 (6)	9.2 (5)	—† (1)	4.0 (14)	1.5 (16)
50–59.....	8.1 (1)	16.2 (1)	129.8 (12)	113.5 (7)	61.8 (21)	13.2 (11)
60 and over.	191.4 (13)	153.9 (7)	77.1 (7)	185.3 (42)	160.2 (69)	55.8 (19)
All ages.....	21.0 (16)	15.3 (14)	34.7 (24)	54.6 (50)	31.7 (104)	7.4 (46)
	Pipes and other types					
Less than 50.	1.4 (3)	4.0 (24)	5.7 (25)	3.8 (1)	4.1 (53)	1.5 (16)
50–59.....	129.4 (4)	16.2 (5)	20.4 (17)	45.3 (14)	27.0 (40)	13.2 (11)
60 and over.	0.0 (0)	132.3 (6)	352.4 (8)	82.7 (30)	88.2 (44)	55.8 (19)
All ages.....	7.6 (7)	13.5 (35)	14.9 (50)	21.8 (45)	15.3 (137)	7.4 (46)
	Cigarettes and/or cigars (no pipes)					
Less than 50.	2.3 (22)	2.6 (74)	2.5 (49)	2.4 (2)	2.5 (147)	1.5 (16)
50–59.....	13.9 (6)	34.3 (18)	24.9 (53)	38.3 (26)	27.3 (103)	13.2 (11)
60 and over.	43.9 (1)	14.6 (1)	267.1 (6)	38.2 (26)	42.8 (34)	55.8 (19)
All ages.....	7.6 (29)	9.7 (93)	9.9 (108)	12.9 (54)	9.9 (284)	7.4 (46)

*Numbers in parentheses indicate number of lip cancer cases on which rate is based.
†No case in controls.

rates have not been computed for the "mixed" smokers because of the lack of the large numbers of cases required to make the many subdivisions of the data necessary to accomplish this satisfactorily.

As Levin, Goldstein, and Gerhardt (*3*) have pointed out, however, duration of smoking is related to age. To take account of the possibility that the relationship between risk of cancer and duration of smoking, as shown in Appendix Table A, may be a simple function of age, the data for cancer of the lung and of the lip have been reassembled and rates estimated in three age groups divided according to duration and type of smoking.

For cancer of the lung these data are given in table 8. Among those smoking cigarettes only, patients within the same age group do show a slight tendency toward higher rates for the longer duration of smoking. This tendency, however, is irregular and a larger series of cases is necessary to test it adequately. For those smoking cigarettes in combination with other types the same tendency is not apparent, though again the numbers of cases available in each class are too small for reliable conclusions.

Similar data for cancer of the lip are given in table 9. When age at diagnosis is kept constant there is no evidence that increased duration of

pipe smoking increases risk of this type of cancer. Again, however, the numbers of cases available are too few to permit reliable conclusions.

These data, however, do appear sufficient to raise some question as to the significance heretofore attached to duration of smoking. Until a large enough series of cases is available to take account of age, it is not possible to determine whether or not the apparent relationship is real or is an artifact due to the age of the patients.

Methods and Findings Compared With Those of Other Studies

Sources of Data

As noted previously, the studies of Schrek, Baker, Ballard, and Dolgoff (*1*), Wynder and Graham (*2*), Levin, Goldstein, and Gerhardt (*3*), Doll and Hill (*4*), and Mills and Porter (*5*), as well as the present one, all employed the familiar case-history method. Patients interviewed for retrospective history of smoking were those coming to specified hospitals in all but two studies—Wynder and Graham (*2*), and Mills and Porter (*5*). In the former, private patients were also included, and in the latter, lists of names for interview of survivors were obtained from death certificates. Interviews were conducted either by trained lay interviewers or by physicians, except in the study of Mills and Porter (*5*) who mailed questionnaires to survivors of lung-cancer decedents. In *none* of these studies, including the present one, can the patients interviewed be regarded as completely representative samples of all lung cancer occurring in definable populations.

The types of individuals selected for comparison ("controls") varied, though in all but one study they consisted essentially of patients with illnesses other than lung cancer. The major exception, that of Mills and Porter (*5*) consisted of residents of Columbus, Ohio, interviewed in house-to-house visits.

Definitions of Smokers

Smokers were defined in each of the six studies as follows:

a) Schrek *et al.* (*1*) defined cigarette smoking as the use of 10 or more cigarettes per day; "moderate" smokers being those using 10–20 cigarettes, and "heavy," those smoking more than that. They did not consider pipe or cigar smoking.

b) Wynder and Graham (*2*) grouped smokers in 5 classes (light smokers—those using 1 to 9 cigarettes per day for more than 20 years—up to chain smokers, who used 35 or more per day for at least 20 years). Pipe and cigar smokers were included by counting 1 cigar as 5 cigarettes and 1 pipeful as 2½ cigarettes.

c) Levin *et al.* (*3*) included those who "had ever smoked," with no specification of quantities used. They also provided tabulations for cigar and pipe smokers.

d) Doll and Hill (*4*) defined as smokers those using one or more cigarettes daily for as long as a year. Their tabulations provide data for five

different quantities smoked daily. Pipe tobacco is converted to the tobacco equivalent of cigarettes.

e) Mills and Porter (*5*) did not report their definition of smokers.

f) The present study classes as smokers those who smoked one or more cigarettes a day,[7] and provides several groups according to quantities. For "mixed" smokers, pipe tobacco and cigars are converted to tobacco equivalents in cigarettes.

General Findings

The major findings of Schrek *et al.* (*1*) are summarized in the following table:

Type of illness	Number of white male patients	Percentage of moderate and heavy cigarette smokers
Controls (tumors other than respiratory or upper digestive tract)	486	50. 0
Lung cancer	73	*71. 2
Cancer of the larynx and pharynx	69	*69. 6
Cancer of the lip	116	**62. 9

* Significantly higher than controls — P=.01.
** Higher than controls — P=.01 — .05.

When the data were age-standardized the excess of smokers in the lung cancer patients over that among controls was of borderline significance (P=.03); that of larynx and pharynx was still significantly greater (P=.01), while the excess among lip cancer cases was no longer significant (P=.06).

Wynder and Graham (*2*) presented their data in considerable detail with regard to the age of patients and controls; the several different types of controls and methods of interview; and the quantity smoked and the duration of smoking. Their general finding, however, is shown in the following table:

Class of patient	Number of patients	Percentage with history of all forms of smoking combined
Lung cancer	605	98. 7
Control	780	85. 3

This difference is significant at a probability level of P=.01.

[7] Included among smokers were a few patients who smoked less than one cigarette per day. However, they oked some such quantity as one pack of cigarettes per month regularly.

Levin, Goldstein, and Gerhardt (*3*) presented their results in two general ways. The first of these, in terms of the percentages of smokers in each class of patients, may be summarized as follows:

Class of patient	Number of patients	Any type	Cigarettes	Pipes	Cigars
Lung cancer	236	84. 7	66. 1	13. 5	11. 2
Other cancer (except lip)	666	82. 9	*48. 0	*25. 8	*20. 3
Lung nontumors	124	81. 1	53. 1	25. 5	13. 4
Other noncancer	481	78. 3	*44. 1	*25. 3	*22. 7

* Significantly different from lung cancer at a probability level of $P=.01$.

Their data also showed a significantly higher proportion of pipe smokers among patients with cancer of the lip than found either among the other cancer patients (except lung cancer) or among other noncancer patients.

By utilizing the smoking histories of all male patients at Roswell Park Memorial Institute during the period 1938–1948, these authors were also able to calculate the proportion of lung and lip cancer cases among smokers and nonsmokers in the hospital patient population. Through this procedure an estimate of the relative risk of these cancers was calculated. Thus, the ratio of the prevalence among smokers of cigarettes to that among nonsmokers was 2.4 for lung cancer. The same ratio was 2.3 for pipe smokers in the lip cancer group. The differences on which both of these ratios are based are statistically significant.

Doll and Hill (*4*) directed considerable attention to the adequacy of their sample of cases and controls and to the duplicability of smoking histories obtained. They considered duration of smoking, daily and total quantities used, and type of smoking.

Their results, in terms of percentages of smokers in cases and controls, are summarized below:

Diagnosis	Type of smoking				
	All forms combined		"Pure" smoking		
	Number	Percent	Number	Percent "pure" cigarette	Percent "pure" pipe
Lung cancer	649	99. 7	525	94. 3	5. 7
Diseases other than cancer	649	95. 9	507	90. 3	9. 7

These differences are highly significant statistically.

These authors also estimated the relative risk of lung cancer among smokers and nonsmokers on the assumption that the smoking histories of the controls were representative of those of the population of Greater

London. Estimates so made are presented for several age classes by quantity of tobacco consumed daily (their table XIV).

Mills and Porter's (*5*) findings, in terms of the percentage of smokers in each class, are as follows:

Class of patient	Number of patients	Percentage of smokers		
		Smokers of cigarettes only	Pipe, cigar or combination smokers	Nonsmokers
Lung cancer	444	55.0	37.0	7.0
Controls	430	43.0	26.0	31.0

With regard to lung cancer, therefore, all of the studies cited showed a significant association between smoking and that disease. In addition, Schrek and co-workers noted an association between cigarette smoking and cancer of the larynx and pharynx; and Levin and co-workers an association between pipe smoking and cancer of the lip.

Comparisons in Terms of Relative Risk of Lung Cancer

With the data at hand, it is possible to compare in more detail the present study with those of Wynder and Graham (*2*) and Doll and Hill (*4*).

Prevalence rates of cancer of the lung per 100,000 white males were estimated for smokers and nonsmokers from the data recorded in these three studies. These rates, calculated by the Cornfield method (*7*), are given in table 10 for several age classes by quantities smoked. For the tabulations shown, cigars and pipe tobacco have been converted to their tobacco equivalents in cigarettes, and total rates are standardized for age for each study. This table also shows the relative prevalence of lung cancer among smokers and nonsmokers.

It will be noted that in each study the groupings differ slightly according to quantities smoked. In addition, different factors are used for conversion of pipe tobacco and cigars into their cigarette equivalents. These variations in classification reduce in detail the comparisons which are possible. It should be noted also, that in spite of the relatively large number of cases and controls in each study, the numbers available are small for computation of rates in many age and smoking classes.

In spite of these considerations several facts appear evident in the comparisons shown. First, it is clear in all three studies that the estimated rate is significantly greater in smokers than in nonsmokers. The relative prevalence, however, varies from 5 in the present study to 14 in the others. Second, among smokers there is a general tendency manifest in all studies toward an increase in rates with increased quantities smoked daily. Some peculiarities in this tendency are manifest, however. In both the Wynder and Graham and the present study, this tendency is quite irregular for patients of 60 years of age and over

TABLE 10.—*Comparisons of findings in three studies. Prevalence rates of cancer of the lung per 100,000 males by age of patients and quantity smoked.*

	Number of patients		Estimated prevalence rates per 100,000 males							Relative* prevalence
	Lung cancer	Control	Quantity smoked daily†							
			1–4	5–14	15–24	25–49	50 or more	All quantities	Non-smokers	
A. Doll and Hill (*4*):‡										
Age:										
30–39	24	24	1.7	3.1	3.4	5.5	–§	3.5	0.0	.....
40–49	122	122	10.9	13.0	15.9	31.2	31.4	16.2	0.0	.....
50–59	276	275	24.5	34.2	41.9	76.7	93.8	41.9	7.1	5.9
60–69	197	199	35.8	51.9	62.8	116.0	137.2	63.0	0.0	.....
70–74	28	27	18.9	53.3	58.5	114.8	–§	62.5	0.0	.....
All ages:										
Crude	647	647	7.7	10.9	13.2	24.4	31.4	13.3	0.9	14.8
Standardized‖	.....	.....	23.4	34.0	41.1	76.4	83.6	41.4	3.0	13.8
			Quantity smoked daily							
			1–9	10–15	16–20	21–34	35 or more	All quantities	Non-smokers	
B. Wynder and Graham (*2*):										
Age:										
30–39	14	146	4.4	2.8	3.5	6.8	0.0	3.6	1.8	2.0
40–49	105	164	3.0	3.1	12.3	31.7	54.6	17.2	0.0	.....
50–59	258	210	9.1	17.0	33.6	131.1	116.9	46.6	4.4	10.6
60–69	187	160	3.6	40.1	80.4	174.8	138.5	70.1	4.7	14.9
70–79	41	100	54.5	67.4	106.3	66.2	84.7	75.5	5.6	13.5

All ages:										
Crude	605	780	2.8	6.9	12.8	31.1	31.6	14.8	1.1	13.5
Standardized ‖			8.3	22.0	43.8	113.0	103.1	46.2	3.4	13.6
			Quantity smoked daily							
C. The present study:			1–10	11–20	21–30	31–40	41 or more	All quantities	Non-smokers	
Age:										
30–39	17	22	2.2	2.5	7.3	8.7	0.0	3.7	2.2	1.7
40–49	167	271	8.6	15.4	15.5	34.7	32.0	16.7	4.7	3.6
50–59	198	208	22.3	40.7	54.2	50.7	87.2	44.5	12.5	3.6
60–69	76	71	33.1	89.5	171.0	84.3	44.2	70.9	5.1	13.9
70–79	14	20	49.9	27.5	124.8	0.0	110.7	62.2	41.2	1.5
All ages;										
Crude	472	592	7.3	12.7	17.1	20.9	21.1	14.0	4.0	3.5
Standardized ‖			22.7	46.1	78.4	52.2	61.5	44.7	9.8	4.6

*Relative prevalence is the ratio: rate among smokers/rate among nonsmokers.

†Quantity smoked daily is all forms of smoking expressed as cigarettes, with pipes and cigars converted to their equivalents in cigarettes as follows:—A. Doll and Hill—1 oz. of tobacco a week=4 cigarettes a day; B. Wynder and Graham—1 cigar=5 cigarettes, 1 pipeful=2½ cigarettes; and C. the present study—1 cigar=10 cigarettes, 1 oz. pipe tobacco=20 cigarettes.

‡The exact number of patients in each age-quantity group was not available in the Doll and Hill paper (4). Therefore, the number of patients used in each group here was estimated from their tables II and V.

§No case in controls.

‖Age-standardized with the three-study combined lung cancer population as the standard population. Note: This standardization yields rates for this table that are different from those obtained in table 5.

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for the larger quantities smoked. At least a part of such discrepancies is due to the small number of cases available in these classes. In the Doll and Hill study the discrepancies noted for the older ages and larger quantities smoked are not apparent.

Discussion

It is quite evident from this study, as well as the others discussed, that an association exists between smoking and cancer of the lung among the cases interviewed. It is also clear that cigarette smoking is the principal form responsible for the demonstrated association. Any of these or other similar studies, taken alone, would hardly justify the conclusion that the association is real, since it is evident that none of them qualify as comprising representative samples of all lung cancer occurring in males from any definable population. Since, however, a number of studies conducted by slightly different techniques, in different geographic regions, and among varying patient populations all point in the same direction, it appears highly likely that the association is not only statistically significant, but also real.

Complete confidence in its genuineness, and more precise measurement of its degree are not likely to be attained, however, by further repetition of this type of investigation. General experience attests to the practical difficulties involved in obtaining a representative sample of cases, or in selecting and interviewing a completely adequate control group for comparison. Such difficulties are as likely to appear in future case-history studies as have been evident in these. Population studies—rather than case-history studies—projected into the future, are probably required for more complete testing of this association and for a more precise estimate of its degree. Judging, however, by the accumulated evidence to date it would be surprising if such studies did not generally confirm the findings of this and previous investigations.

It should be pointed out that an association between cancer and some type of smoking has been demonstrated or suggested for each site under review here, except cancer of the pharynx. The question may therefore be raised as to whether this evidence indicates an association between smoking and cancer in general, rather than between it and these specific sites. The data of Schrek, Baker, Ballard, and Dolgoff (*1*) and Levin, Goldstein, and Gerhardt (*3*), utilizing large series of "other sites" as controls, do not support an hypothesis of a general association. Their grouping of a number of sites, however, might conceivably have masked existing associations for some of the individual sites they included.

Assuming that present evidence justifies the conclusion that cigarette smokers are more likely to develop lung cancer than nonsmokers, the question remains as to whether the smoking of cigarettes is of itself responsible for the increased risk. Other authors have presented evidence in conformity with the view that cigarette smoking has weak carcinogenic effects and is thus of important etiological significance.

Certain observations in this study are not necessarily consistent with that view.

It is common practice to note the association between cigarette smoking and lung cancer and relate this observation to the parallel increase in cigarette consumption and lung cancer during recent years. In this study an association of about the same degree as that observed with cancer of the lung has been shown between cancer of the larynx and cigarette smoking. A phenomenal increase in cancer of the larynx, such as that recorded for cancer of the lung, has not been observed in this country. If the association between cigarette smoking and lung cancer is of etiological significance some explanation is needed as to why a corresponding increase in laryngeal cancer has not followed an increase in cigarette consumption, with which this disease is also associated.

These data also provide no evidence that age at onset of disease is influenced by the quantities of cigarettes smoked. While the lack of such evidence may be due to an insufficient number of cases, no suggestion of an increased average age at onset with decrease in cigarettes used, was evident. This point needs testing in other series since one might expect such a correlation if cigarettes were of etiological significance.

In the same fashion these data provide no firm evidence that risk increases with increase in duration of smoking. When age was kept constant no clear-cut trend in this direction was apparent. As pointed out, however, the number of cases available in this series is insufficient to test this point adequately. Levin, Goldstein, and Gerhardt (*3*) recognized a possible independent association between age at onset and duration of smoking but, since their smokers of less than 25 years' duration were few in number, it is not clear whether or not their method of testing accounted for age adequately.

It would therefore appear that though the association between cigarette smoking and lung cancer seems real, the significance of this association remains unestablished.

Summary and Conclusions

Retrospective smoking histories of 1,990 patients with cancer of the lip, tongue, other oral cavity, pharynx, esophagus, larynx, and lung were analyzed and compared with those of 615 patients with illnesses other than cancer. Comparisons were made of percentages of smokers in each group, as well as in terms of the magnitude of relative risk of cancer among smokers and nonsmokers. The findings were also compared with those of several other recently recorded studies.

The magnitude of the relative risk of cancer at each site among smokers and nonsmokers was measured by converting the proportions of smokers in test and control groups into prevalence rates of cancer. This conversion is dependent upon the assumptions that the smoking history of the controls is representative of the smoking history of the noncancer population, and that the smoking histories for each cancer site are representative of all cancer at such sites.

An association between smoking and cancer was considered to be demonstrated in this series of cases if all of the following conditions were met:

1. The rate in smokers was significantly higher than in nonsmokers at a probability level of $P = .01$ or less;
2. There was a fairly good direct relationship between quantities smoked and estimated rates;
3. The age-specific rates in smokers of a particular type exceeded and generally paralleled those estimated for nonsmokers; and
4. That for "mixed smokers" rates were excessive among those practicing the type of smoking under suspicion, though in combination with another type.

On the basis of these general criteria it is concluded that the following associations are demonstrated in this series: *a*) between pipe smoking and cancer of the lip, *b*) between cigarette smoking and laryngeal cancer, and *c*) between cigarette smoking and cancer of the lung.

Additional associations are suggested but are not believed to be demonstrated in this series, namely: *a*) between pipe and cigar smoking and cancer of the tongue, *b*) between pipe and possibly cigar smoking and other cancer of the oral cavity, and *c*) between cigarette smoking and cancer of the esophagus.

There was no suggestion at all of any association between pharyngeal cancer and smoking of any type.

Since previous studies of a similar nature have also demonstrated associations between these types of smoking and cancer of the lip, larynx, and lung, it is concluded that these associations are real.

The magnitude of the relative risk of lung cancer among smokers and nonsmokers, which has been demonstrated in this study, has been compared to that found in two other studies. In this study the rate in smokers was estimated to be 5 times that in nonsmokers, while in the other two it was 14.

Aside from the statistical significance and apparent realness of these associations there remains the question of whether smoking is etiologically related to lung cancer. Considerations are presented which lead to the conclusion that the etiological significance of these associations remains unestablished.

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APPENDIX TABLE A.—*Estimated crude prevalence rates of cancer per 100,000 white males by site of cancer, type of smoking, and duration of smoking*

Site of cancer and type of smoking	Duration of smoking in years						
	Less than 10	10–19	20–29	30–39	40–49	50 and over	All durations
Lip:							
Cigarettes only	4.4	9.1	10.4	9.4	13.1	11.8	9.9
Cigars only	4.5	7.5	9.9	3.4	53.2	0.0	8.1
Pipes only	21.8	20.6	15.3	35.0	41.3	67.3	31.7
Tongue:							
Cigarettes only	2.1	2.7	3.7	6.7	7.1	6.2	5.2
Cigars only	0.0	8.9	0.0	31.2	63.6	0.0	11.9
Pipes only	0.0	4.5	5.2	20.9	25.9	46.6	16.7
Oral Cavity:							
Cigarettes only	5.2	2.5	3.9	7.7	19.3	11.5	7.3
Cigars only	19.2	13.8	28.9	19.4	78.6	9.6	21.1
Pipes only	6.5	16.5	14.5	38.7	48.1	57.7	30.1
Pharynx:							
Cigarettes only	2.1	3.4	2.8	1.7	4.0	3.1	2.5
Cigars only	0.0	0.0	0.0	0.0	31.4	0.0	1.5
Pipes only	0.0	2.2	2.6	3.4	5.1	2.6	2.9
Esophagus:							
Cigarettes only	3.2	4.2	8.3	8.2	17.8	19.1	9.0
Cigars only	0.0	0.0	23.9	0.0	145.6	0.0	11.4
Pipes only	0.0	0.0	0.0	5.3	31.8	23.8	8.9
Larynx:							
Cigarettes only	1.2	3.2	6.6	10.0	20.3	10.9	9.1
Cigars only	6.1	0.0	0.0	9.1	36.9	9.1	5.2
Pipes only	0.0	0.0	3.0	4.0	15.1	12.1	5.5
Lung:							
Cigarettes only	4.4	4.3	10.3	14.6	25.9	31.2	13.7
Cigars only	5.5	2.4	16.5	12.4	0.0	16.2	8.6
Pipes only	10.9	0.0	1.4	12.8	8.2	0.0	5.0

Addendum

Since this paper was accepted for publication, analyses have been completed of the smoking histories obtained from 1,091 white male patients with cancer of the skin who were interviewed in the same hospitals as those considered above. Skin cancer prevalence rates were estimated for smokers and nonsmokers to test the possible existence of an association with smoking for a nonrespiratory site of cancer. Utilizing the same procedures employed in the body of this report, and accepting the previously described general criteria as evidence, no association between smoking and skin cancer could be demonstrated.

The general results may be summarized as follows:

Type of smoking	Estimated prevalence rates of skin cancer per 100,000 white males of all ages	
	Crude rate	Age standardized
Cigarettes only	*4. 4	5. 2
Cigars only	5. 6	6. 6
Pipes only	*16. 8	11. 4
Mixed	*5. 6	5. 7
All smokers	*5. 8	6. 4
Nonsmokers	9. 6	10. 3

*Significantly different from the rate in nonsmokers at a probability level of P=.01 or less.

Though the crude rate among pipe smokers is significantly higher than that among nonsmokers, this difference disappears when the rates are standardized for age. However, no explanation can be given for the apparently lower rates for smokers of other types. It is therefore apparent that this series of cases provides no evidence for the existence of an association between smoking and cancer in general. In addition, these results provide further reason for believing that the associations demonstrated in the body of this report, as well as in other studies, are real.

After this paper went to press, Doll and Hill[8] published an extension and elaboration of their previous findings. It should be pointed out that their method of estimating relative risk in smokers and nonsmokers is similar to that employed here. The magnitude of their rates, however, is substantially higher than those estimated here because Doll and Hill use, as their base, mortality rates for lung cancer in Greater London which materially exceed those recorded in the United States. All rates in the present study, including those computed for comparison with the data of Wynder and Graham and those of Doll and Hill's first report, were based on prevalence rates as estimated for the United States in 1937. The relative risks between smokers and nonsmokers, as derived by the two methods, should be the same, though no direct comparisons can be made since Doll and Hill's latest study employed different age groupings of patients.

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Lesions Induced in Mice by Painting With *p*-Dimethylaminobenzene-1-Azo-2-Naphthalene [1,2]

AMBADAS S. MULAY *and* ERKKI A. SAXÉN,[3]
National Cancer Institute,[4] *Bethesda, Md.*

The carcinogenicity of azo compounds similar to *p*-dimethylaminoazobenzene is being studied in this laboratory. The carcinogenicity of *p*-dimethylaminobenzene-1-azo-1-naphthalene, in male Osborne-Mendel rats, has been established (*1*). *p*-Dimethylaminobenzene-1-azo-2-naphthalene (DA-2-N) was fed to rats by Kinoshita (*2*) to test its carcinogenicity, but he found no neoplastic lesions. The present paper deals with the production of neoplastic lesions with *p*-dimethylaminobenzene-1-azo-2-naphthalene (DA-2-N). Hemangioendothelioma and other non-neoplastic lesions induced in C57BR mice by painting with DA-2-N solution are described in this paper.

Materials and Methods

Fifty-seven C57BR mice (27 males and 30 females) 30 to 45 days old, were painted between the shoulder blades with a 1 percent solution of *p*-dimethylaminobenzene-1-azo-2-naphthalene. Benzene and polyethylene glycol 600 were used as solvents for the DA-2-N azo dye. The animals in Group 1 (12 males and 13 females) were painted with the benzene solution and those in Group 2 (15 males and 17 females) with the polyethylene glycol solution of DA-2-N. All mice were painted 3 times a week for 39 weeks, a total of 117 applications. Assuming that about 0.1 ml. of the solution was painted on the skin at each application, each mouse received a total calculated dose of about 120 mg. of the carcinogen. All mice were fed Purina laboratory chow pellets throughout the experiments.

Seven mice were autopsied 2 to 3 months after the beginning of the experiment, and 50 mice were autopsied at 8 to 14 months. The animals killed after 8 months had developed large tumors or appeared moribund. Tissues from all animals were fixed in Zenker's acetic or Zenker's formol

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[2] The lesions were: hemangioendothelioma and hyperplastic and neoplastic lesions of the liver, and hydronephrotic lesions of the kidney.

[3] *Present address:* Department of Pathology, Central Institute for Radiotherapy, Helsinki, Finland.

[4] National Institutes of Health, Public Health Service, Federal Security Agency.

fluids. Microscopic sections were stained routinely with hematoxylin and eosin. Some sections were prepared by Wilder's silver method for reticulum.

Results

In the seven animals killed between 2 and 3 months after the start of the experiment, no significant lesions were found, except for vascular dilation at the site of the painting. This may have been an inflammatory response or a preliminary stage in the development of the hemangioendotheliomas found later.

In the 50 mice (27 males and 23 females) that were killed between 8 and 14 months after the start of the experiment, the significant lesions found were hemangioendothelioma at the site of painting, hyperplastic and neoplastic lesions of the liver, and hydronephrotic lesions. Table 1 classifies these lesions according to solvent used and the duration of the experiment.

Hemangioendothelioma

Forty-eight mice developed tumors of this type. On inspection the tumors varied from a few millimeters to 3 centimeters in diameter, and averaged about 1 centimeter. On section many of these tumors exuded a bloody fluid, and usually only a small amount of residual firm tissue remained after this fluid drained away. In some animals the tumors had ulcerated before the animals were killed, and at autopsy they appeared superficially as large areas covered with a thin surface of bloody exudate.

Upon histologic examination, the seven mice examined in the shorter period after the start of the experiment showed slight hyperkeratosis of the epidermis in some areas. In other areas the epidermis was thin, consisting of a single flattened layer of epithelial cells. The dermis under the painted areas was thickened and was more eosinophilic than dermis in other areas (figs. 1, 2). The subcutis was edematous and contained blood-filled spaces lined with endothelium consisting of one layer of cells (fig. 2). Fine capillary vessels, endothelial-lined tubes, nonluminized cords of endothelial cells, fibroblasts and small areas of infiltration with lymphocytes, plasma cells, and endothelial cells (fig. 3), were also found in subcutis and muscle. Severe degenerative changes were observed in the underlying muscle tissue. Many muscle fibers had lost their striations and appeared hyalinized. The number of nuclei in these areas appeared to be reduced. Some necrotic cells were found in the fat tissue. Histologic evidence was not sufficient to determine whether this rich vascular tissue may represent a true angioma or whether it may be due to a reparative process. Damage to the walls of the blood vessels may have resulted from the toxic factors in the substance used for painting and may have led to secondary dilatation of the vessels. A new growth of capillaries arising probably as a reactive process to hemorrhages or anoxia was also present. All these changes are entirely in keeping with an inflammatory response; however, these changes may have been forerunners in the development of hemangioendotheliomas found later.

TABLE 1.—*Hemangioendotheliomas and hepatic and renal lesions of mice painted with 1% solution of* p-*dimethylaminobenzene-1-azo-2-naphthalene*

Solvent	Duration of experiment	Number of mice	Hemangioendothelioma at the site of painting	Liver lesions				Kidney
				Amyloidosis	Bile-duct tumors	Hypertrophic hepatic-cell lesion	Hepatoma	Hydronephrosis
	(*days*)							
(1) Benzene	233–297	12	12	3	2	5	2	2
Benzene	303–312	4	4	4	0	2	2	2
Total		16	16	7	2	7	4	4
(2) Polyethylene glycol	233–297	12	12	0	0	4	1	5
Polyethylene glycol	303–312	8	8	0	0	2	2	3
Total		20	20	0	0	6	3	8
(3) Polyethylene glycol	317–408	14	12	2	0	4	2	3
Total for all experiments		50	48	9	2	17	9	15

Fully developed hemangioendotheliomas were found at or near the site of painting in 48 and in pararenal tissue in 3 mice killed at the later period. These tumors fitted the description of hemangioendothelioma in the mouse given by Edwards *et al.* (*3*). The tumors were usually situated in the fat tissue below the panniculus carnosus, but may also appear more superficially in the subcutaneous fat.

Histologically, all of the tumors were essentially similar. Blood-filled vessels and solid sheets of cells were closely intermingled. The vascular spaces were lined with an endothelial type of cell, highly variable in size and chromatin content. Frequently the cells projected deeply into the lumen. The pleomorphic cells forming the solid sheets and cords resembled those lining the vascular channels (fig. 4). Giant cells having a single nucleus were common. The distribution of reticulum in these tumors was characteristic of hemangioendothelioma. In 9 cases, the tumors appeared to be composed of a purely vascular tissue of capillary and cavernous blood-filled spaces lined by slightly swollen endothelial cells that projected into the lumen. In 5 tumors, the cells forming the solid sheets were fusiform, and some areas of them resembled fibrosarcoma.

The tumors were not encapsulated. The tissues adjacent to the tumor often showed evidence of hemorrhage and necrosis of fat cells with inflammatory-cell infiltration. Small hemangioendotheliomas and angiomatous areas were present in many cases near the largest tumors and suggested multicentric foci, rather than extension from a single tumor. No other form of sarcoma and no carcinomas were found at the site of painting.

Lesions in the Liver

The livers of 50 mice that died or were killed between 233 and 408 days, were examined histologically. Areas of localized hypertrophy of liver cells were recognized, and this lesion has been distinguished from fully developed hepatomas.

Some of the lesions developing in the livers of these mice were remarkable for the apparent involvement of epithelium of the bile ducts. They were unlike the lesions ordinarily seen after the ingestion of other agents that produce hepatomas in mice. These lesions resembled cholangiomas and bile-duct changes described in rats by Mulay and Firminger (*1*) and in mice by Cook *et al.* (*13*). For purposes of tabulation these liver lesions have been designated as hyperplastic hepatic-cell lesions, hepatomas, hypertrophic biliary changes, and bile-duct tumors.

Hyperplastic biliary changes, ranging from a simple localized increase in the number of ducts in the portal area to diffuse growth of atypical biliary tissue were seen in 18 livers. In 14 cases there were newly formed bile ducts lined with cuboidal or columnar epithelium in the periportal areas. In 2 cases, the biliary cells had lost their normal arrangement and formed solid areas without lumina (figs. 5, 6). These are listed in the table as bile-duct tumors. A few specimens showed slight perivascular round-cell infiltration. Amyloidosis was found in 9 cases.

Development of tumors diagnosed as hepatomas was observed in 9 cases. The nodules were all nonencapsulated, but they compressed the adjacent liver tissue where condensed reticulum was found (fig. 7). The cells and their condensed arrangement had a striking resemblance to that of liver parenchyma. The cords were usually one to two cells thick, alternating regularly with endothelial-lined blood sinuses. The endothelium did not show any active proliferation. Scattered remnants of bile ducts were occasionally seen in the periphery. The cells of the typical hepatoma had prominent margins and were usually much larger than normal hepatic cells. The cytoplasm was finely granular and, in many cases, cells were also found with large vacuoles resembling hydropic degeneration. The cytoplasm was faintly basophilic in most nodules. The nucleus was of the same size as the nucleus of normal hepatic cells, or perhaps larger, with one to three prominent nucleoli. Mitotic figures were occasionally found, as well as occasional multinucleated and mononucleated giant cells. Eosinophilic, spherical cytoplasmic inclusions about the size of a nucleus were observed in 5 cases (4).

It should be pointed out that there was a gradation between hepatomas and the hypertrophic foci of hepatic cells so that there was not always a distinct point of separation between them. However, some division seemed desirable in this study.

Lesions of the Kidney

Evidence of hydronephrosis was found in 15 of the 50 mice studied. In 11 cases, it was unilateral and on the right side. In 4 cases, both kidneys were affected. On inspection, the kidney was greatly increased in size and consisted of a thin shell of tissue containing a vesicle of clear fluid (fig. 9). The ureter did not appear to be dilated.

Remarkably little or no change in the cortex was seen in most of the cases. In some, however, the cortical tubules and Bowman's spaces showed dilatation (fig. 8). Pyelitis and pyelonephritis, which are so common in human hydronephrosis, were rarely seen. Amyloidosis or necrosis of the papilla was not observed. The normal contralateral kidney seldom showed compensatory hypertrophy. No sign of inflammatory disease of the renal pelvis was noted.

Hemangioendotheliomas were found in the pararenal tissue in three mice (fig. 10). The tumors were typical hemangioendotheliomas and were similar to tumors found in subcutaneous fat. In two cases, the tumors grew around the ureter and might have caused blockage of the ureter (fig. 11). Hydronephrosis was present in these three cases.

Other Changes

In two mice, multiple pulmonary adenomas were found. Except for the changes in the liver and kidney, no other changes were found in the internal organs.

Discussion

There is a voluminous literature on tumors produced by painting carcinogenic agents on the skin of mice. Hartwell (*5*) records over 500 such papers. Carcinogens used include anthracenes, phenanthrenes, chrysenes, benzpyrenes, cholanthrenes, fluorenes, steroids, and azo compounds. However, reports of the production of hemangioendothelioma at the site of painting by any of these compounds are rare. Hartwell and Stewart (*6*) report the induction of hemangioendothelioma in 4 percent of the mice painted with 5,9,10-trimethyl-1,2-benzanthracene. No hemangioendotheliomas induced by painting with azotoluene or azobenzene compounds are described in the literature.

Many investigators have induced hemangioendotheliomas in several strains of mice by administering various carcinogens by subcutaneous injection (*7, 8*), implantation (*9*) or oral feeding (*10*). White and Stewart (*10*) produced hemangioendotheliomas in 30 percent of C3H mice and 45 percent of C strain (BALB/c) mice by feeding methylcholanthrene in the diet, and Andervont (*8*) produced the tumor in as high as 67 percent of the C strain (BALB/c) mice by subcutaneous injection of *o*-aminoazotoluene.

In the present study, painting with *p*-dimethylaminobenzene-1-azo-2-naphthalene, produced hemangioendotheliomas in 96 percent of the mice that survived 240 days after the start of the experiment. These tumors at the site of painting were located in the interscapular and subcutaneous fat tissue. It is of interest to note that this predilection for fatty tissue was observed by other workers. Andervont (*8*) who induced hemangioendotheliomas in mice by subcutaneous injection of *o*-aminoazotoluene, observed that most of these tumors were within fat deposits in the interscapular region, abdomen or subcutaneous tissue, although these tumors were found at a variety of sites distant from the site of injection.

Many investigators, among them Law (*11*), Kirby (*12*), Cook (*13*), and Andervont (*14*), produced characteristic hepatomas in mice by either painting with, or subcutaneous injection of, azo compounds. The liver lesions produced by painting with *p*-dimethylaminobenzene-1-azo-2-naphthalene are remarkable in producing a growth of epithelium resembling that of bile ducts, and the production of tumors resembling cholangioma. Cook *et al.* (*13*) report such lesions in the liver of mice treated with azonaphthalene.

Damage to the liver parenchyma was found in almost all cases and appeared as an increase in intralobular reticulum and connective tissue. Hepatomas were found in 9 of the 50 mice. Most of the tumors were well differentiated and resembled spontaneous hepatomas of mice or hepatomas induced by *o*-aminoazotoluene.

Dunn observed (*15*) that congenital anomalies of the kidney are infrequent in inbred strains of mice commonly used at the National Cancer Institute. There are no published reports of kidney anomalies in C57BR mice. However, evidence of hydronephrosis was observed (*16*) in this laboratory in about 20 percent of C57BR mice receiving 9,10-dimethyl-1,2-benzanthracene. These mice can be considered as controls for the

DA-2-N experiments, as they came from the same stock, had the same age and sex distribution, and were under observation at the same time. Unilateral hydronephrosis in 22 and bilateral in 8 percent of the mice treated is of interest, as this is the first time hydronephrosis of unknown etiology has been reported in a high percentage of C57BR mice. Remarkably few parenchymal changes were seen in the kidney and evidence of pyelitis or pyelonephritis was only occasionally observed. Amyloidosis or necrosis of the papilla were also absent. The etiology of this lesion is obscure. As this lesion is not characteristic of any effect so far described as due to the action of a chemical agent, it may be a congenital anomaly in this strain of mice.

In these experiments there were no controls for the solvents used. However, other investigators have painted the skin of various species of animals, including mice, with benzene and no neoplasm was found at the site of application, nor any liver lesion discovered by any of the investigators (*5*). Group 1 differs from group 2 only in the solvent used; therefore, the differences found between these two groups could be attributed to the solvent used.

The experimental groups No. 1 and No. 2, in which benzene and polyethylene glycol, respectively, were used as solvent for DA-2-N, showed some noteworthy differences in liver and kidney pathology. The liver changes were slightly more marked in the benzene group, as judged by the increase of reticulum and connective tissue and the hypertrophic hepatic-cell lesions. Amyloidosis of the liver was also present in 7 of the 16 mice in the benzene group but in none of the 20 mice in the group that received polyethylene glycol solution. Hepatomas occurred in 4 of the 16 mice in the first group and in 3 of the 20 mice in the second group. All mice developing hepatomas in the benzene group were males, and all mice with hepatomas in the second group were females. Two tumors with marked growth of biliary cells appeared in the benzene group.

The two groups, one of which received DA-2-N in benzene solution and the other one in polyethylene solution, are small and do not justify far-reaching conclusions. However, a comparison between them indicates that DA-2-N in benzene solution damages liver more than DA-2-N in polyethylene glycol solution and perhaps also induces hepatomas and proliferation of biliary cells more frequently.

Summary

p-Dimethylaminobenzene-1-azo-2-naphthalene (DA-2-N) when painted as a 1 percent solution, either in benzene or polyethylene glycol 600, produced hemangioendotheliomas in subcutaneous fat tissue at the site of painting. Degenerative changes and hyperkeratosis were observed in the epidermis of the mice sacrificed 60 to 90 days after the start of the experiment, and 48 out of 50 mice that survived 240 days developed hemangioendotheliomas. In 3 mice, hemangioendotheliomas were discovered in pararenal fatty tissue, a site far distant from the site of painting.

Liver lesions of varying severity, from hypertrophy of small groups of hepatic cells to distinct hepatomas, were observed in 52 percent of the mice that survived 240 days. Thirty-five percent of these lesions were diagnosed as hepatomas. These liver lesions tended to be more severe in the mice painted with benzene solution of the carcinogen than ones painted with polyethylene glycol 600 solution. Two cholangiomas were observed in these DA-2-N painted mice.

Hydronephrosis was observed in 38 percent of the mice that lived over 8 months after the start of the experiment. This lesion was more common in males than in females, and was usually unilateral. However, the etiology of this lesion and the role, if any, of DA-2-N in its production are obscure.

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PLATES

PLATE 137

Lesions in C57BR mice painted with p-*dimethylaminobenzene-1-azo-2-naphthalene. All sections are from the site of painting. All are stained with hematoxylin and eosin.*

FIGURE 1.—The dermis is thickened and the subcutis edematous. Fine capillary vessels, endothelial-lined tubes, cords of endothelial cells, and slight lymphocytic infiltration are seen. × 220

FIGURE 2.—The dermis is thick and dense. Blood-filled spaces appear in the subcutis. × 220

FIGURE 3.—Blood-filled spaces, fine capillary vessels, endothelial-lined tubes and lymphocytic infiltration below the site of painting in the subcutis and muscle layer. × 260

FIGURE 4.—DA-2-N induced hemangioendotheliomas of interscapular fat. × 260

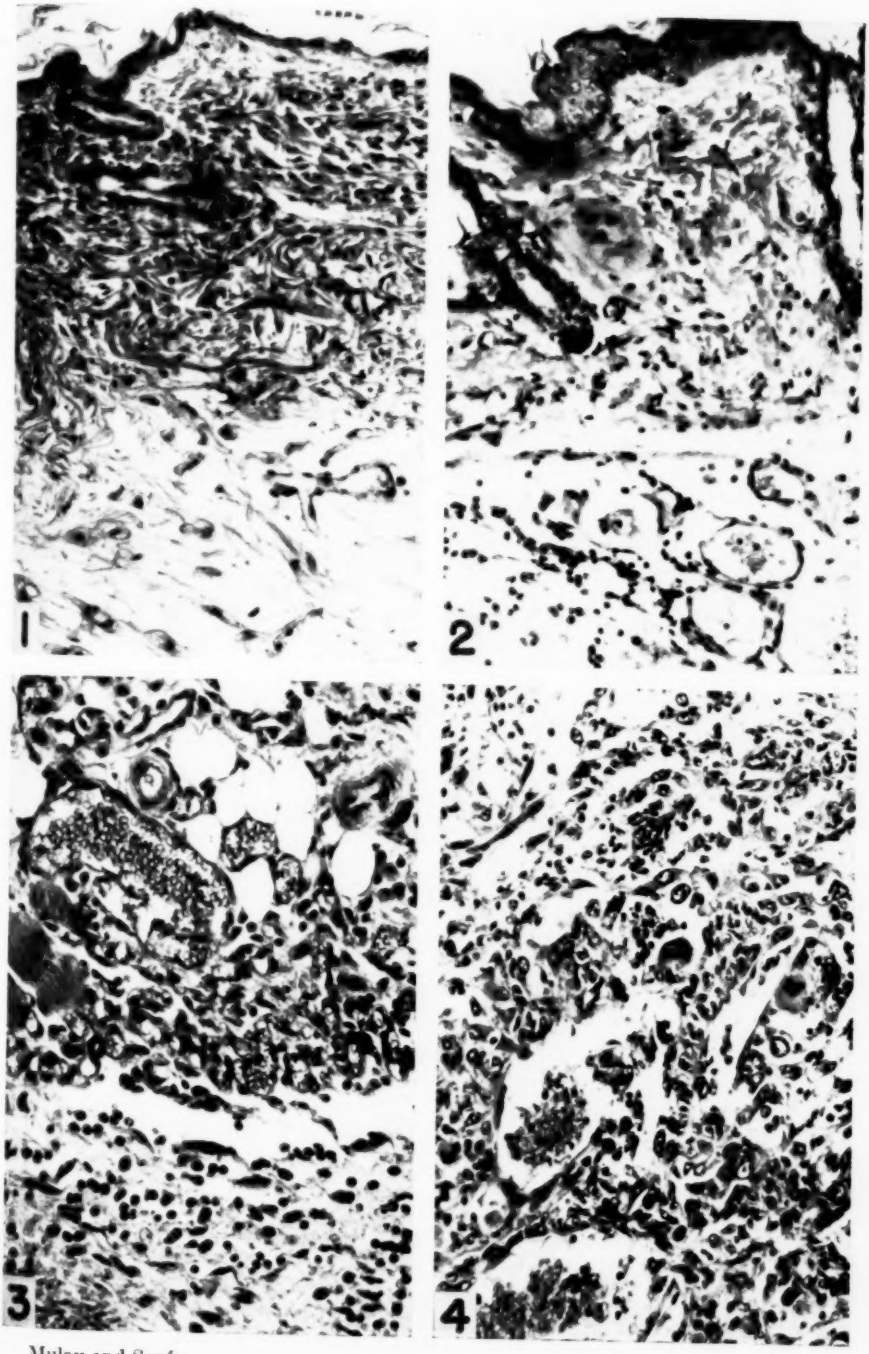

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PLATE 138

Liver tumors of mouse that was painted for 275 days with 1 percent DA-2-N in benzene; no painting for 107 days.

All sections are stained with hematoxylin and eosin.

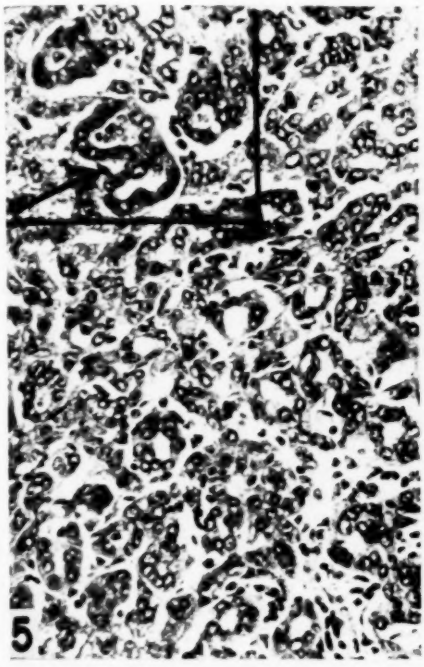
FIGURE 5.—Diffuse growth of cells resembling bile-duct epithelium. The nuclei are large and the cells resemble hepatic cells, except for basophilic cytoplasm and arrangement in single cell layers about a lumen. × 260

FIGURE 6.—Detail of section from figure 5. × 610

FIGURE 7.—DA-2-N in polyethylene glycol. Induced hepatoma and adjacent compressed hepatic tissue. × 260

Kidney changes in C57BR mice following painting with DA-2-N.

FIGURE 8.—Dilatation of cortical tubules and Bowman's spaces in a hydronephrotic kidney. × 260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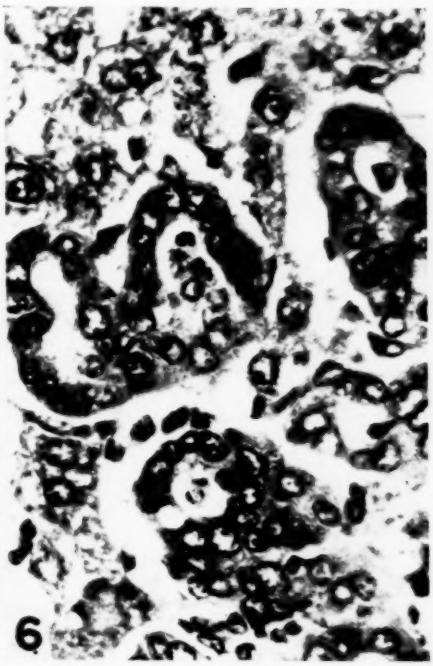
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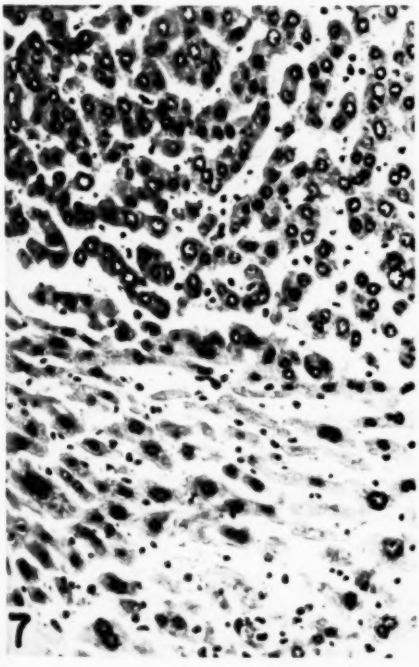
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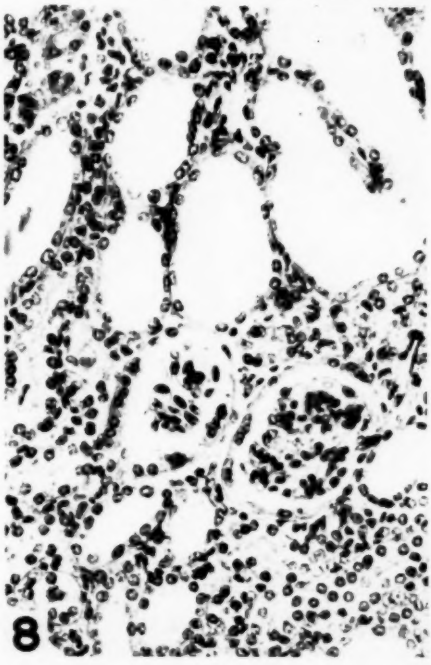
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PLATE 139

Kidney changes in C57BR mice following painting with DA-2-N.
All sections are stained with hematoxylin and eosin.

FIGURE 9.—Thin shell of kidney cortex, with remnant of papilla, showing vesicle once filled with clear fluid. × 9

FIGURE 10.—Hemangioendothelioma developing at the surface of the kidney and in the pararenal fat tissue. × 29

FIGURE 11.—Hemangioendothelioma in the periureteral fat tissue. The lumen of the ureter appears in the center. × 260

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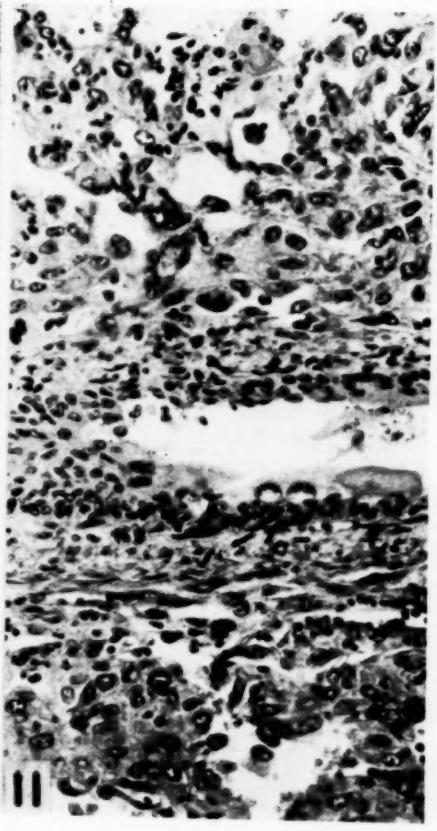

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The Survival of the Mammary Tumor Milk Agent in Cultures of Heterologous Cells [1, 2]

MARY A. PIKOVSKI,[3] *Department of Experimental Pathology (Cancer Laboratories), Hebrew University, Jerusalem, Israel*

Several animal viruses not pathogenic for the chicken have been cultivated *in vitro* in chicken-embryo tissue (*1*), but comparatively little is known about the capacity of such viruses to grow in specific or unspecific cell types. In respect to tumor-producing viruses, this problem has been variously interpreted; Carrel (*2*, *3*) maintained that the virus of Rous Sarcoma I can be propagated in chicken monocytes but not in fibroblasts, whereas Ludford (*4*) succeeded in growing this virus in chicken fibroblasts. The transformation of normal fibroblasts into tumor cells *in vitro* under the influence of this virus was subsequently established by Halberstaedter, Doljanski and Tenenbaum (*5*).

Similarly in the case of the virus of chicken leukemia, Furth and Breedis (*6*) claimed that "oncogenic viruses multiply *in vitro* only in the presence of cells on which they confer neoplastic properties," while Doljanski and Pikovski (*7*, *8*) were able to maintain a strain of virus of chicken leukemia (hemocytoblastosis) for long periods *in vitro* by serial cultivation of the virus in chicken fibroblasts, though the cells specific for this strain are the hemocytoblasts on which unrestricted proliferation is induced by the action of the leukemic agent *in vivo*.

There are no data as yet on the possibility of cultivation *in vitro* of the milk agent of mouse mammary carcinoma, though several attempts have been made to demonstrate the presence of this virus in the yolk of embyronated eggs in which mammary tumors were grown. Taylor *et al.* (*9–11*) succeeded in some experiments in producing carcinomas and sarcomas at the site of injection of filtered or dried yolk of such eggs. Neither Heilmann and Bittner (*12*) nor Twombly and Meisel (*13*) were able to confirm such tumor-inducing properties of cell-free yolk, but the presence of virus in the yolk, infected 12 days previously with a virus-containing material, was later demonstrated by Bittner *et al.* (*14*).

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In the present investigation, the possibility was explored of maintaining the milk agent in cultures of chicken fibroblasts—cells belonging to a cell type nonspecific for the activity of this agent.

Materials and Methods

Preparation of Extracts Containing the Milk Agent

In six experiments, the initial fibroblast cultures were infected with filtered extracts of mammary tumors from RIII strain mice, and in two experiments with centrifuged extracts of lactating mammary glands from normal females of the same strain.

Tumor extracts.—Spontaneous tumors 3 to 4 weeks old were freed from necrotic and hemorrhagic parts, minced, and triturated with Ringer's solution. This fine, 10 to 15 percent tumor suspension was kept at 4° C. for 1 hour and then centrifuged at 3,000 revolutions per minute for 15 minutes. The supernatant fluid was passed through a Boerner centrifugal filter 5114B (A. H. Thomas & Co.), using Seitz filter discs of asbestos and cellulose fibers (supplied by the same company). These filters were tested and proved to be bacteria-proof, to insure that no tumor cells could be present in the filtrates. The filtration was performed in the centrifuge at 3,000 revolutions per minute. This method prevented foaming, and was therefore adequate for the filtration of the tumor virus material, as well as convenient for the small amounts used.

Mammary gland extracts.—Mammary glands from a mouse that had nursed her second or third litter for 14 to 16 days were minced, triturated, suspended in 3 parts Ringer's solution, and centrifuged in conical tubes for 30 minutes. The thick cream was carefully removed, and the supernatant fluid was transferred to another tube and centrifuged again for 15 minutes. If any visible sediment was noted, the supernatant fluid was centrifuged for 15 minutes more. For the excision and extraction of tumors and mammary glands, ice-cooled instruments and glassware were used, and the tubes were surrounded by ice during centrifugation. Any mouse cells that might conceivably have persisted in the supernatant fluid would probably not have proliferated in the media used; their presence would, in any case, not have prejudiced the experiment.

The infectivity of the extracts used for infecting cultures of chicken fibroblasts was tested by the subcutaneous inoculation of 0.5 ml. of each extract into backcross hybrids (see section on Test Animals). These tests also served as a control of the susceptibility of the backcrosses to the milk agent.

Tissue Cultures

Cultures of chicken fibroblasts, grown from either adult or embryonic chicken hearts, were excised and kept for 2 hours at 4° C. in a cell-free extract containing the milk agent. The infected cultures were planted in D-3.5 Carrel flasks, containing a solid phase of 1 ml. of Tyrode's solution, 0.5 ml. of chicken plasma, and 1 drop of diluted chick-embryo extract for coagulation of the plasma. A similar embryo extract was added as

a fluid phase and usually taken off after 24 hours, and the majority of the cultures remained without fluid phase until transplantation. The cultures were washed with Tyrode's solution, fed with diluted chick-embryo extract every third day, and transplanted after 12 to 16 days (or earlier, if necessary).

For transplantation, the cultures were excised, bathed in Ringer's solution, each divided in half, planted in the same media as the initial cultures, and subsequently dealt with in the same way.

Eight series of cultures, each derived from a different set of initial normal fibroblast cultures and infected by a different virus-containing extract, were cultivated. Each series comprised several serial passages (transplant generations).

The activity of the virus in cultures of each passage was tested by inoculating mice with the remainder of the contents of the Carrel flasks, after the excision of the cultures used for transplantation. For this purpose, the solid and, if present, the fluid phase, were finely minced, triturated, diluted 1:1 with Ringer's solution, and inoculated subcutaneously in the region of the mammary glands of the backcross test mice. Two or three days after the first inoculation, parallel cultures of the same passage were inoculated into the same mice using similar methods, so that each animal received one to three inoculations of cultures of the same passage. The period of cultivation of the injected cultures of each passage, as recorded in table 1, is the age of the cultures of the first inoculation. In some cases, cultures of two consecutive passages were inoculated into the same mice (table 1).

Test Animals

The RIII and C57BL strains of mice used for these experiments were obtained through the courtesy of Dr. Samuel Graff, of the College of Physicians and Surgeons, Columbia University, in 1947 and since bred in this laboratory.

The inoculations for the test on the activity of the virus were made on backcross hybrids obtained by crossing C57BL females to RIII males and backcrossing the BR F_1 hybrids to RIII males.

The daughters of some of their litters were used for inoculations. In other litters, the daughters were bred to their brothers and their progeny were also used for inoculations.

In two experiments, seven C57BL mice were also used for inoculations. Three to five suckling females, 10 to 18 days of age and all belonging to one or two litters, were used for each test.

The mice were returned after the inoculation to their respective mothers. They were weaned at the age of 4 weeks and bred to their brothers—their progeny were discarded a few days after birth.

Mice having the same parents, but from a previous litter or from the one following the litter used for inoculation, were left as uninjected control animals.

All the mice were kept 4 to 6 to a cage, fed with Purina laboratory chow pellets, and received tap water *ad libitum*.

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TABLE 1.—*Inoculation into mice of chicken fibroblast cultures infected with the milk agent*

Experiment No.	Period of cultivation (*days*)	Passage	No. of mice inoculated	No. of tumor-bearing mice	Average latent period of tumors (*months*)	Non-tumor-bearing mice: average age at death (*months*)
1	19	1	*4	1	11	22
"	30	2	*3	1	13	7. 5
"	53	3	2	2	8	.....
2	12	1	3	1	11	15. 5
"	21	1	same mice.	.....	.....	.....
"	37	2	4	1	19	16
"	52	3	2	0	.....	22. 5
"	69	4	3	2	16	6. 5
"	82	5	same mice.	.....	.....	.....
"	91	6	2	1	11	9. 5
"	106	7	3	1	7	8. 5
"	122	8	same mice.	.....	.....	.....
"	140	9	4	2	15	15. 5
"	156	10	same mice.	.....	.....	.....
3	39	2	3	2	15	22. 5
"	49	3	same mice.	.....	.....	.....
"	58	4	3	1	14	17. 5
"	72	5	same mice.	.....	.....	.....
4	20	1	4	1	12	8. 5
"	28	2	4	4	4	.....
"	41	3	4	2	20. 5	16
"	56	4	same mice.	.....	.....	.....
5	11	1	4	0	.....	20
"	47	4	3	1	8	16. 5
"	75	8	4	2	12	11. 5
"	86	9	same mice.	.....	.....	.....
"	96	10	3	3	13. 6	.....
6	10	1	4	3	10. 7	11. 5

*In these two experiments the C57BL mice were used.

Results

Of eight groups of inoculation experiments with eight series of cultures, six groups were positive, the presence of an active agent being demonstrated in cultures of most of the passages in these series. The inoculations of the other two groups were entirely negative.

The inoculation of the six extracts used for the infection of the initial cultures of the six positive series, resulted in mammary tumors in 11 of 23 treated mice (47.8%) after an average period of 12 months.

No tumors developed in mice inoculated with the two extracts used for the infection of the initial cultures of the two negative series, but the absence of the agent in these extracts was not definitely established, since not all the inoculated mice lived to tumor age.

The results of the inoculations of the six positive groups are summarized in table 1, showing that the inoculation of cultures into 66 mice resulted in tumors in 31 mice (2 of them in C57BL mice), representing a 47 percent tumor yield.

It is interesting to note that positive results were obtained up to the tenth passage in 2 groups of experiments (cultivated for 96 and 156 days,

respectively), though negative results were observed in earlier passages in the same groups (see experiments 2 and 5).[4] In experiments 1, 3, and 4, cultures of 3 to 5 passages were inoculated and positive results were obtained with each passage. Experiments 5 and 6 are not complete, owing to the loss of a large number of mice during a recent epidemic. The true number of positive results might have been higher, since a considerable number of mice died before reaching tumor age. The percentage of tumor incidence was lowest in the mice inoculated with cultures of the first passage; it did not differ much between the second and eighth passages, and it was highest in the ninth to tenth passages (table 2). The latent period (the time between the inoculation and the first appearance of the tumor) varied considerably in individual mice inoculated with cultures of earlier as well as of later passages. The average latent period for the tenth passage corresponded to that for the third passage.

TABLE 2.—*Mammary tumor incidence in mice inoculated with different serial passages of chicken fibroblast cultures infected with the milk agent*

Passage	No. of mice inoculated	No. of tumor-bearing mice	Average latent period of tumors (*months*)	Tumor incidence (*percent*)
1	19	6	11.1	31
2	14	8	12.7	57
3	8	4	14.2	50
4–5	9	4	12.6	45
6–8	9	4	10	45
9–10	7	5	14.3	71
Total	66	31	12.4	47

All the tumors appeared in the region of the mammary glands, though not always at the exact site of inoculation, after an average latent period of 12.4 months following inoculation of the cultures. The tumors often showed a characteristic type of growth: after reaching the size of a lentil or a pea the tumor tended to regress, especially during pregnancy. However, 2 to 4 weeks later it reappeared at the same site, and when it began to develop steadily more tumors appeared on other mammary glands, so that the mouse ultimately died with three or more tumors.

Histologically these tumors were mammary carcinomas of variable type, showing a restricted range of modification of a basic acinar structure which, as described by Kirschbaum and Bittner (*15*), Kirschbaum, Williams, and Bittner (*16*), Huseby and Bittner (*17*), and by Andervont and Dunn (*18*), is typical for the tumors produced by the milk agent.

Neither squamous metaplasia nor sarcomatous changes of the stroma—which besides the acinar type sometimes appear in mice without the participation of the milk agent—were ever observed in our treated mice. (Four atypical cases of a different nature will be discussed elsewhere.)

[4] After this paper was submitted for publication, the one survivor of the group inoculated with cultures of experiment 5, 12th passage (112 days of cultivation) developed a mammary carcinoma

Discussion

The inoculation of tumor or mammary gland extracts that had been used for the infection of the initial cultures, resulted in tumors in 47.8 percent of the mice tested. This result gives an approximate measure of the susceptibility of the backcross mice used for the inoculation of cultures of infected fibroblasts. It should be expected, if the cultures contained the agent, that a similar percentage of mice should react to the inoculation of the cultures. In fact, 47 percent of the mice inoculated with extracts of cultures of different passages developed mammary tumors.

Of 96 uninjected control backcrosses that lived to tumor age, 5 developed spontaneous mammary tumors (5.2%) that were histologically of the same basic acinar structure as those in the injected mice. In view of these facts it may be concluded that, though it is possible that a small percentage of tumors that developed in the inoculated mice were of spontaneous nature, the large majority were undoubtedly the result of the inoculation of the cultures containing the active milk agent.

The mouse mammary tumor agent must, therefore, have survived for several months, after many serial passages in cultures of chicken fibroblasts, and remained active as long as these cells were cultivated *in vitro*.

The method of tissue culture in Carrel flasks, though suitable for the cultivation of pure cell strains and therefore adequate for the present investigation, does not lend itself to quantitative evaluation of a virus by titration techniques. The validity of the conclusions drawn is based, however, on the following considerations: *a*) The cultures were divided in each passage, so that practically nothing of the initial cultures remained after 10 passages, and the cultures of each passage were planted in fresh medium and washed before transplantation. Nevertheless the tumor incidence was higher in the tenth than in the first passage, and the latent period was not longer in the later than in the earlier passages (table 2). *b*) The mice that received the inoculations of extracts of cultures developed almost as high a percentage of tumors, after the same average period of time after inoculation, as those inoculated with 0.5 ml. of concentrated extract of tumor or lactating mammary glands. *c*) It was demonstrated by Barnum *et al.* (*19*) that mammary tumor extracts giving 50 percent positive takes after being kept for 1 hour at 4° C. gave only 21 percent takes after 1 hour at 37° C. The agent in the present cultures, however, was active and gave 47 percent takes after 156 days of cultivation at 37.5° C.

These considerations are indicative of actual multiplication of the virus and of the reinfection of the outgrowing heterologous fibroblasts in the cultures of each passage, rather than of a mere survival of the virus absorbed by the cells of the initial culture.

Summary

Cultures of chicken fibroblasts were infected with the milk agent and cultivated in Carrel flasks through 10 serial passages.

Cultures of every passage were tested for virus activity.

The milk agent was found to be active in cultures of chicken fibroblasts as long as these cells were cultivated *in vitro* (156 days).

The tumor incidence was higher in mice inoculated with extracts of cultures of the tenth passage than in those of the first passage.

The inoculation of 0.5 mil. of cell-free extracts of tumor or mammary gland used for the infection of the initial cultures, resulted in tumors in 47.7 percent of the mice tested.

The inoculation of extracts of the infected cultures resulted in tumors in 47 percent of the mice.

It is suggested that not only a survival but a real cultivation of the milk agent took place in the nonspecific heterologous cells.

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Tobacco-Smoking Habits in an American City [1]

CLARENCE A. MILLS, M. D., *and* MARJORIE MILLS PORTER, M. D., *Laboratory for Experimental Medicine, University of Cincinnati, Cincinnati, Ohio*

The world-wide interest in the deleterious effects of tobacco smoke, especially as a possible etiologic factor in tumors of the respiratory tract, makes it imperative that a base-line of smoking habits be established in respect to age, sex and race of standard groups of population. Most studies on the effects of tobacco smoke have utilized sick people (hospitalized or ambulant clinic patients) as controls. This is unsound experimental procedure to determine whether tobacco smoke may be injurious, since it is very probable that tobacco smoking is much more common among such ill persons than among non-hospital cases.

An earlier study of the health aspects of air pollution (*1*) revealed a close relationship of polluted atmosphere and fatal respiratory disease (pneumonia, tuberculosis, and pulmonary tumors) in the cleanest in contrast to the dirtiest districts of various American industrial cities. It seemed to the authors that similar relationships may prevail in the case of air polluted individually by tobacco smoke. In 1947, a survey of Columbus, Ohio, was undertaken to ascertain the smoking habits of a city. Although the character of the smoking habits undoubtedly changes, accurate records relating to sex, race and age from all the census areas of an entire city would be of great value.

Methods

Columbus lists (1940 census) a population of 270,183 white and 35,904 Negro residents, and it is a city of moderate size and industrialization representative of the United States. It was estimated that it would be necessary to interview 8 percent of the Negro residents and about 0.9 percent of the white population in order to ensure adequate numbers for statistical analysis of each category of white and colored men and women. One of the authors (M. M. P.) made house-to-house visits at random to secure smoking data of the required percentage of inhabitants in the 61 census areas of the city. Only residents of 20 years of age or over were selected, and the data were obtained directly from the individual concerned or from a responsible member of the household. The data included

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age, sex, and color, and the type and extent of tobacco smoking of the resident. The following categories were devised for records: *a*) Cigarettes: light (less than 1 pack daily), and heavy (1 or more packs daily); *b*) Cigars: light (less than 3 cigars daily), and heavy (3 or more cigars daily); *c*) Pipe: light (less than 3 pipefuls daily), and heavy (3 or more pipefuls daily); *d*) Cigar and pipe smoking combined; *e*) Cigar and cigarette smoking combined; *f*) Pipe and cigarette smoking combined; *g*) Cigar, pipe and cigarette smoking combined; and *h*) Nonsmoker.

The final analysis of the data required the use of only four of these categories as follows:

1) Cigarettes: light smoker,
2) Cigarettes: heavy smoker,
3) Cigars, pipe or any combination that included either or both, and
4) Nonsmoker.

The case histories as recorded were brief and covered only the specific answers desired, with no effort to cover other factors in the individual's existence. Examples are as follows:

1) Census tract 46: J. D., female, aged 56 years, white, married, interviewed by home house call, smoked 3–5 cigarettes daily for last 17 years, smokes occasional cigar, no pipe.

2) Census tract 56: B. M., male, aged 36 years, white, married, interviewed by home house call, smoked 1½ packs of cigarettes daily for last 17 years, smokes occasional cigar, no pipe.

Results

Table 1 shows the smoking habits of the white male population of Columbus in three city areas roughly determined on the basis of degree of atmospheric pollution. Table 2 gives the data for white women, and table 3 that for Negro men and women. Since there were no soot-fall or other quantitative air pollution data available in Columbus at the time of our survey, the help of the city's smoke abatement department was enlisted in providing us with a division of the city's census tracts into the following three categories on the basis of the degree of air pollution generally prevailing: Census tracts #1 to #10, 25 to 27, 37, and 54 comprise the cleanest areas of the city; tracts 11 to 20, 44 to 49, 55, 56, and 58 to 61 represent areas that are intermediate in respect to degree of atmospheric pollution; while census tracts 21 to 24, 28 to 36, 38 to 43, 50 to 53, and 57 were the areas of worst air pollution.

The primary purpose of this survey was to establish reference records of groups of people, by age decade, sex, color, and city habitat (cleanest, intermediate and dirtiest areas), whose smoking habits may be compared with that of other groups of similar nature. Such habits may also be related to specific disease in other groups of people. Therefore, an attempt was made to secure adequate numbers for statistical analysis of all categories of smoking habit, and thus a representative sampling of the whole population in respect to age, sex, color and habitat was not necessarily obtained. A comparison of the surveyed with the total

population may be derived from table 6. The 2,456 white persons for whom smoking habit data were obtained were distributed relatively evenly over the range of age decades (over 20 years), and their geographic distribution in respect to air pollution prevailing closely resembled that of the total white population. Only a negligible number of Negroes resided in the cleanest and intermediate areas of Columbus.

Tables 4 and 5 present the material of tables 1 and 3 rearranged so that all smoking combination categories as well as the cigarette category are classified under "cigarettes." In tables 1 and 3, such combinations were listed under the pipe-cigar category. Nicotine is absorbed in greatest amount from inhaled cigarette smoke, and most habitual cigarette smokers inhale. Hence, the cigarette element of combination smokers should be emphasized in studies of disorders such as coronary disease and peptic ulcer in which absorbed nicotine may play an important role.

To be certain that the statistics of the 1947 Columbus survey would serve as valid controls in studies of the smoking habits of people in other cities, a comparative study of one of the smoking categories of residents of Cincinnati was made (*2*). In 1948, the smoking habits of 507 white women of Cincinnati, 20 to 29 years of age, were secured by letter questionnaire. Their names were obtained from the list of those who gave birth to a first child during January and August of 1948. Of these 507 women of Cincinnati, 41.35 percent were smokers (all cigarettes), whereas 39.68 percent of the 310 white women of Columbus in the same age group were smokers. This is not a significant difference. A greater number of persons with various diseases smoke than do residents of Columbus (*2–5*) as shown in table 7.

Discussion

It is evident from the data presented that cigarette smoking in Columbus is most common in young adult persons, and less common in older people. The reverse is true of cigar and pipe smoking, which are most common in older people. Cigarette smoking is greatest in the poorer sections of the city, and least in the cleaner suburban areas, while pipe and cigar smoking are most common in the suburban areas and least common in the poorer sections. The smoking habit may be related to economic status, cigars being the most expensive type of tobacco. Cigarette smoking is more common among Negroes than among white residents of a residential area with the same degree of air pollution.

The increase in percentage of nonsmokers of advancing age (20 to 50 percent for white men and from 58 to 100 percent for white women) is quite definite and significant. It is probably due to a greatly increased incidence in recent years of cigarette smoking among younger people. Consumption of cigarettes increased about tenfold during the period between World War I and World War II. The unwary reader might conclude that change in smoking habits would indicate a loss of the

TABLE 1.—*Tobacco-smoking habits of white men in Columbus, Ohio**

Types of smoking	20–29 years		30–39 years		40–49 years		50–59 years		60 and over	
	No.	Percent	No.	Percent	No.	Percent	No.	Percent	No.	Percent
Clean Districts of the City										
Cigarettes:										
Light	8	10. 26	4	6. 25	2	3. 23	3	5. 08	0	0
Heavy	37	47. 44	29	45. 31	33	53. 23	25	42. 37	5	10. 42
Pipe, cigars or combinations	12	15. 38	16	25. 00	15	24. 19	16	27. 12	27	56. 25
Nonsmokers	21	26. 93	15	23. 44	12	19. 35	15	25. 42	16	33. 33
Total numbers	78	100. 00	64	100. 00	62	100. 00	59	100. 00	48	100. 00
Intermediate Districts of the City										
Cigarettes:										
Light	20	11. 36	4	5. 71	10	10. 64	5	7. 81	9	11. 54
Heavy	96	54. 55	39	55. 71	54	57. 44	28	43. 75	11	14. 10
Pipe, cigars or combinations	22	12. 50	11	15. 71	15	15. 96	16	25. 00	28	35. 90
Nonsmokers	38	21. 59	16	22. 86	15	15. 96	15	23. 44	30	38. 46
Total numbers	176	100. 00	70	100. 00	94	100. 00	64	100. 00	78	100. 00
Dirtiest Districts of the City										
Cigarettes:										
Light	15	15. 46	7	12. 50	3	5. 00	6	9. 23	3	3. 66
Heavy	57	58. 76	33	58. 93	29	48. 33	30	46. 15	21	25. 61
Pipe, cigars or combinations	4	4. 13	5	8. 93	12	20. 00	13	20. 00	16	19. 51
Nonsmokers	21	21. 65	11	19. 64	16	26. 67	16	24. 62	42	51. 22
Total numbers	97	100. 00	56	100. 00	60	100. 00	65	100. 00	82	100. 00

	Whole City									
Cigarettes:										
Light	43	12.25	15	7.89	15	6.95	14	7.45	12	5.77
Heavy	190	54.13	101	53.16	116	53.70	83	44.15	37	17.79
Pipe, cigars or combinations	38	10.83	32	16.84	42	19.44	45	23.93	71	34.13
Nonsmokers	80	22.79	42	22.11	43	19.91	46	29.47	88	42.31
Total numbers	351	100.00	190	100.00	216	100.00	188	100.00	208	100.00

	White Men 60 Years of Age and Over					
	60–69 years		70–79 years		80 years and over	
	No.	Percent	No.	Percent	No.	Percent
Cigarettes:						
Light	9	7.50	3	4.35	0	0
Heavy	32	26.67	6	8.70	1	5.26
Pipe, cigars or combinations	37	30.83	21	30.43	9	47.37
Nonsmokers	42	35.00	39	56.52	9	47.37
Total numbers	120	100.00	69	100.00	19	100.00

*All combination smokers here listed under "Pipe, cigars or combinations."

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TABLE 2.—*Tobacco-smoking habits of white women in Columbus, Ohio*

Types of smoking	20–29 years		30–39 years		40–49 years		50–59 years		60 and over	
	No.	Percent	No.	Percent	No.	Percent	No.	Percent	No.	Percent
Clean Districts of the City										
Cigarettes:										
Light	19	23. 75	15	20. 55	9	13. 85	6	8. 45	5	5. 95
Heavy	14	17. 50	21	28. 77	14	21. 54	4	5. 63	0	0
Nonsmokers	47	58. 75	37	50. 68	42	64. 61	61	85. 92	79	94. 05
Total numbers	80	100. 00	73	100. 00	65	100. 00	71	100. 00	84	100. 00
Intermediate Districts of the City										
Cigarettes:										
Light	13	10. 48	16	16. 49	7	7. 69	6	7. 06	6	5. 00
Heavy	28	22. 58	25	25. 77	10	10. 99	3	3. 53	3	2. 50
Nonsmokers	83	66. 94	56	57. 74	74	81. 32	76	89. 41	111	92. 75
Total numbers	124	100. 00	97	100. 00	91	100. 00	85	100. 00	120	100. 00
Dirtiest Districts of the City										
Cigarettes:										
Light	19	17. 92	12	15. 79	7	10. 15	8	11. 43	6	6. 52
Heavy	30	28. 30	15	19. 74	12	17. 39	9	12. 86	5	5. 43
Nonsmokers	57	53. 78	49	64. 47	50	72. 46	53	75. 71	81	88. 05
Total numbers	106	100. 00	76	100. 00	69	100. 00	70	100. 00	92	100. 00

	Whole City									
Cigarettes:										
Light	51	16. 45	43	17. 48	23	10. 22	20	8. 85	17	5. 74
Heavy	72	23. 23	61	24. 80	36	16. 00	16	7. 08	8	2. 70
Nonsmokers	187	60. 32	142	57. 72	166	73. 78	190	84. 07	271	91. 56
Total numbers	310	100. 00	246	100. 00	225	100. 00	226	100. 00	296	100. 00

	White Women 60 Years and Over					
	60–69 years		70–79 years		80 years and over	
	No.	Percent	No.	Percent	No.	Percent
Cigarettes:						
Light	15	8. 33	2	2. 22	0	.....
Heavy	6	3. 33	1	1. 11	0	.....
Nonsmokers	159	88. 34	88	96. 67	25	100. 00
Total numbers	180	100. 00	91	100. 00	25	100. 00

TABLE 3.—*Tobacco-smoking habits of Negroes in Columbus, Ohio**

Types of smoking	*Men by Age Groups*									
	20–29 years		30–39 years		40–49 years		50–59 years		60 and over	
	No.	Percent	No.	Percent	No.	Percent	No.	Percent	No.	Percent
Cigarettes:										
Light	47	19. 03	29	15. 59	33	17. 01	23	13. 14	10	6. 71
Heavy	141	57. 08	109	58. 60	92	47. 43	67	38. 29	26	17. 45
Pipe, cigars or combinations	15	6. 07	24	12. 90	42	21. 65	52	29. 71	70	46. 98
Nonsmokers	44	17. 82	24	12. 91	27	13. 91	33	18. 86	43	28. 86
Total numbers	247	100. 00	186	100. 00	194	100. 00	175	100. 00	149	100. 00
	Women by Age Groups									
Cigarettes:										
Light	83	28. 33	49	22. 58	28	16. 47	20	12. 42	5	3. 60
Heavy	68	23. 21	49	22. 58	27	15. 88	9	5. 59	4	2. 88
Pipe, cigars or combinations					1	0. 59	1	0. 62	3	2. 16
Nonsmokers	142	48. 46	119	54. 84	114	67. 06	131	81. 37	127	91. 36
Total numbers	293	100. 00	217	100. 00	170	100. 00	161	100. 00	139	100. 00

*All combination smokers here listed under "Pipe, cigars, or combinations."

smokers by death, but such a conclusion (while it may be true) cannot be derived from the data of the present paper.

Since tobacco is advertised on a national scale, it seems reasonable that the only significant regional differences in the smoking habits in America would be those related to age, sex, color and socio-economic status as studied in the Columbus survey. Careful consideration should be given to all these factors in utilizing the Columbus smoking habit statistics in comparative studies. Standard or probable errors have not been included in the tables of basic statistics, since each particular group or groups would require a new calculation of standards in relation to the particular study.

In view of the significantly higher incidence of tobacco smoking in the patients with various diseases (*2–5*) in comparison to that of properly selected segments of the population of Columbus (table 7), it is clear that hospitalized or ambulant clinic patients should *not* be utilized as normal control subjects in any analysis of the relationships of disease and smoking habits. To do so may minimize unwittingly the importance of such studies. It would seem that the results of previously published investigations of the effects of tobacco smoke may have been more significant had they been obtained with valid control cross-section of the total population. Unfortunately, many papers on this subject do not list the age-distribution of control groups, and it thus would be impossible to check the smoking habits of their patients against analogous control segments of the Columbus population. In studies of the age distribution of pulmonary tumors, the smoking habits of the patients were compared with those of an analogous age-sex-color-distribution group of residents of Columbus (table 7). The sharp increase in statistical significance is apparent in each case.

A population of hospital patients should not be utilized as controls in the statistical evaluation of relationships of environment to disease unless the study and control groups are to be limited strictly to certain well-defined classifications. Strong selection factors undoubtedly segregate unclassified hospital patient populations at the outset from the outside population-at-large. Hospital patients possess an inherent, although unknown, bias when used in any type of statistical control study. Wynder (*6*) stated, "We believe, however, that a general hospital population reflects rather closely the general population, particularly since our data [on smoking habits] were not based on the present status of the patient but rather on his status during the past decades at which time he was a member of the general population." This statement does not seem to take into consideration the statistical bias arising from the potent factors that had already operated to separate the hospital patient from the general population-at-large. Indeed, our own preliminary findings seem to indicate that possibly significant factors in the hospitalization of patients with respiratory or circulatory disorders (and in certain other categories) are associated with their past tobacco-smoking habits.

TABLE 4.—*Tobacco-smoking habits of white men in Columbus, Ohio**

Types of smoking	20–29 years		30–39 years		40–49 years		50–59 years		60 and over	
	No.	Percent	No.	Percent	No.	Percent	No.	Percent	No.	Percent
Clean Districts of the City										
Cigarettes:										
Light	9	11. 54	6	9. 38	3	4. 84	3	5. 08	4	8. 33
Heavy	44	56. 41	38	59. 37	38	61. 29	30	50. 85	8	16. 67
Pipe, cigars or combinations	4	5. 13	5	7. 81	9	14. 52	11	18. 64	20	41. 67
Nonsmokers	21	26. 92	15	23. 44	12	19. 35	15	25. 42	16	33. 33
Total numbers	78	100. 00	64	100. 00	62	100. 00	59	100. 00	48	100. 00
Intermediate Districts of the City										
Cigarettes:										
Light	22	12. 54	5	7. 14	10	10. 64	6	9. 37	9	11. 54
Heavy	110	62. 50	44	62. 86	58	61. 70	31	48. 44	14	17. 95
Pipe, cigars or combinations	6	3. 41	5	7. 14	11	11. 70	12	18. 75	25	32. 95
Nonsmokers	38	21. 59	16	22. 86	15	15. 96	15	23. 44	30	38. 46
Total numbers	176	100. 00	70	100. 00	94	100. 00	64	100. 00	78	100. 00
Dirtiest Districts of the City										
Cigarettes:										
Light	15	15. 46	7	12. 50	5	8. 33	7	10. 77	3	3. 66
Heavy	61	62. 89	38	67. 86	38	63. 33	34	52. 31	25	30. 49
Pipe, cigars or combinations	0	0. 00	0	0. 00	1	1. 67	8	12. 31	12	14. 63
Nonsmokers	21	21. 65	11	19. 64	16	26. 67	16	24. 62	42	51. 22
Total numbers	97	100. 00	56	100. 00	60	100. 00	65	100. 00	82	100. 00

	Whole City									
Cigarettes:										
Light	46	13. 11	18	9. 47	9	8. 80	16	8. 51	16	7. 69
Heavy	217	61. 82	123	64. 74	134	62. 03	95	50. 53	47	22. 60
Pipe, cigars or combinations	8	2. 82	7	3. 68	20	9. 26	31	16. 49	57	27. 40
Nonsmokers	80	22. 79	42	22. 11	43	19. 91	46	24. 47	88	42. 31
Total numbers	351	100. 00	190	100. 00	216	100. 00	188	100. 00	208	100. 00

	White Men 60 Years of Age and Over					
	60–69 years		70–79 years		80 yrs. and over	
	No.	Percent	No.	Percent	No.	Percent
Cigarettes:						
Light	9	7. 50	6	8. 70	0	0
Heavy	42	35. 00	7	10. 17	1	5. 26
Pipe, cigars or combinations	27	22. 50	17	24. 64	9	47. 37
Nonsmokers	42	35. 00	39	56. 52	9	47. 37
Total numbers	120	100. 00	69	100. 00	19	100. 00

*Combination smokers using cigarettes here listed under "Cigarettes."

TABLE 5.—*Tobacco-smoking habits of Negroes in Columbus, Ohio**

Types of smoking	*Men by Age Groups*									
	20–29 years		30–39 years		40–49 years		50–59 years		60 and over	
	No.	Percent	No.	Percent	No.	Percent	No.	Percent	No.	Percent
Cigarettes:										
Light	51	20. 65	29	15. 59	39	20. 10	31	17. 71	15	10. 07
Heavy	143	57. 89	113	60. 75	93	47. 94	71	40. 57	27	18. 12
Pipe, cigars or combinations	9	3. 64	20	10. 75	35	18. 04	40	22. 86	64	42. 95
Nonsmokers	44	17. 82	24	12. 91	27	13. 91	33	18. 86	43	28. 86
Total numbers	247	100. 00	186	100. 00	194	100. 00	175	100. 00	149	100. 00

*Combination smokers using cigarettes here listed under "Cigarettes."

TABLE 6.—*Comparison of surveyed with total population*
(by sex, color, age decade, and degree of air pollution)

Age decade	20–29	30–39	40–49	50–59	60 and over	Total	Surveyed in each area as percent of total surveyed (*percent*)	Area population (20 years and over as percent of city total (*percent*)
White males surveyed:								
15 clean census tracts	78	64	62	59	48	311	26. 97	24. 24
22 intermediate tracts	176	70	94	64	78	482	41. 80	38. 52
24 dirty tracts	97	56	60	65	82	360	31. 22	37. 24
All census tracts	351	190	216	188	208	1, 153	100. 00	
White males in general population	23, 293	21, 766	18, 779	14, 956	14, 860	93, 654		100. 00
White females surveyed:								
15 clean census tracts	80	73	65	71	84	373	28. 63	23. 68
22 intermediate tracts	124	97	91	85	120	517	39. 68	41. 00
24 dirty tracts	106	76	69	70	92	413	31. 70	35. 32
All census tracts	310	246	225	226	296	1, 303	100. 00	
White females in general population	25, 809	22, 427	20, 565	15, 817	18, 419	103, 037		100. 00
Colored males surveyed	247	186	194	175	149	951		
" " in general population	3, 090	3, 198	3, 000	1, 983	1, 346	12, 617		
Colored females surveyed	293	217	170	161	139	980		
" " in general population	3, 256	3, 148	2, 656	1, 595	1, 413	12, 068		

TABLE 7.—*Smoking habits of various disease groups vs. appropriate Columbus controls*

Disease groups	Patient groups			Columbus controls			Patient group vs. Columbus controls		
	No. cases	Nonsmokers (*percent*)	Standard error	No. cases	Nonsmokers (*percent*)	Standard error	Difference	Standard error	Difference divided by standard error of difference
White males only:									
Upper resp. cancer deaths (*3*)	124	9. 68	2. 65	185	37. 84	3. 65	28. 16	4. 45	6. 3
Lower resp. cancer deaths (*3*)	444	7. 21	1. 23	430	30. 47	2. 22	23. 26	2. 54	9. 2
Pulmonary tuberculosis, cases (*2*)	517	14. 89	1. 56	777	24. 20	1. 54	9. 31	2. 19	4. 2
Pneumonia, cases (*2*)	118	15. 25	3. 31	472	25. 21	2. 00	9. 96	3. 86	2. 6
Peptic ulcer, cases (*2*)	55	18. 18	5. 20	275	34. 91	2. 88	16. 73	5. 94	2. 8
Coronary heart, deaths (*2*):									
40–49 years	56	7. 14	3. 44	216	19. 91	2. 72	12. 77	4. 39	2. 9
50–59 years	216	6. 66	2. 15	188	24. 47	3. 14	17. 81	3. 80	4. 7
60–69 years	153	18. 30	3. 11	114	35. 09	4. 47	16. 79	5. 44	3. 1
70+ years	130	33. 84	4. 15	88	54. 12	3. 31	20. 28	6. 74	3. 0
Cincinnati maternity cases (*2*)	520	58. 65	2. 16	310	60. 32	2. 78	1. 67	3. 52	0. 5
Wynder & Graham (*4*):	(Diff./S. E. of Diff. = 9.9)								
Lung cancer	605	1. 32	0. 46	605	31. 10	1. 88	29. 78	1. 94	15. 4
Hospital patient controls	780	14. 74	1. 27	605	31. 10	1. 88	16. 36	2. 27	7. 2
Doll & Hill (*5*):									
Lung cancer (males)	649	0. 31	0. 22	649	26. 05	1. 72	25. 74	1. 74	14. 8
Hospital patient controls	649	4. 16	0. 78	649	26. 05	1. 72	21. 89	1. 89	11. 6
	(Diff./S. E. of Diff. = 4.1)								
Lung cancer (females)	60	31. 67	6. 01	600	81. 41	1. 59	49. 74	6. 21	8. 0
Hospital patient controls	60	53. 33	6. 44	600	81. 41	1. 59	28. 08	6. 63	4. 2
	(Diff./ S. E. of Diff. = 2.5)								

Summary

The tobacco-smoking habits of residents (20 years of age and over) in all census tracts of Columbus, Ohio, were studied and classified by age decade, sex, color, and habitat in respect to atmospheric pollution. No significant difference was found between the smoking habits of 20- to 29-year-old white women of Columbus and those of a similar group of women in Cincinnati.

In Columbus, cigarette smoking was found to be more common in poorer dirty sections of the city than in clean suburban areas, whereas the reverse was found in the case of cigar and pipe smoking. Cigarette smoking was more predominant in young adults than in older people, while people of advanced age commonly smoked cigars or pipes. It is emphasized that these well people at their homes, rather than hospitalized or ambulant clinic patients, should be utilized in studies of the relationship of tobacco smoke and disease.

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(*4*) Wynder, E. L., and Graham, E. A.: Tobacco smoking as a possible etiologic factor in bronchogenic carcinoma. J. A. M. A. 143: 329, 1950.

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A Survey of Transplantable and Transmissible Animal Tumors [1]

LUCIA J. DUNHAM, *Medical Officer, and* HAROLD L. STEWART, *Chief, Laboratory of Pathology, National Cancer Institute,*[2] *Bethesda, Md.*

Prepared at the National Cancer Institute under the Auspices of the Sub-Committee on Oncology of the Committee on Pathology of the National Research Council

Foreword

BALDUIN LUCKÉ, M. D., *Chairman, Subcommittee on Oncology*

Animal tumors, propagated in the laboratory by transplantation to suitable hosts, are indispensable material in various phases of cancer research. Such tumors make it possible to have available for many kinds of experimental investigation neoplastic tissue as nearly standardized as is possible with living things. A well-established kind of transplantable tumor in a pure-bred line of animal is to the worker in the field of cancer almost the equivalent of a pure chemical or a pure culture of a microorganism. There is now a large number of such tumors in several species of vertebrates. The need to assemble the widely scattered information about them has long been felt. Thanks to the zeal of Doctors Dunham and Stewart, this has now been accomplished. As modestly stated by them, this is a first attempt and there will no doubt be found some omissions. It seems best not to delay publication, however, but to distribute this survey in the hope that it will be useful to many and that corrections will be made in future editions. Every investigator who uses transplantable tumors is urged to communicate to the authors any information that should be incorporated in future editions. Only through such co-operation can this work be of continued value.

—*Balduin Lucké*

Introduction

The earliest report of a transplantable tumor appeared in 1889. In that year Hanau (*148*) announced the successful intrascrotal transplanta-

[1] Received for publication October 24, 1952.

[2] National Institutes of Health, Public Health Service, Federal Security Agency.

tion of a metastasis from "a spontaneously developing horny cancer of the vulva of a white rat." The rat that had received the tumor implant showed peritoneal carcinomatosis at autopsy. Hanau expressed the opinion that he had transplanted proliferating body cells and that carcinoma was not an infectious disease. In 1894, Morau (*223*) carried out successful transplantation experiments with mouse mammary tumors. He interpreted this as the transference of an agent, and was not aware of the full significance of his experiment. After the turn of the century several investigators, particularly those of the Imperial Cancer Research Fund and of the Rockefeller Institute, and Loeb in 1901 (*197*) and Jensen in 1903 (*161*), worked with spontaneous tumors that proved to be transplantable. The chance finding by Lucké (*202*) of a renal carcinoma in a leopard frog initiated the study of transplantable neoplasms in cold-blooded vertebrates.

The transplantation of animal tumors that had been experimentally produced proved to be possible. In 1920 Fibiger transplanted tumors induced in mice with tar (*106*), and in addition tumors arising in mice infected with spiroptera (*105*). Although the origin of his spiroptera tumors is controversial (*155*), it is certain that his tar tumors in mice were experimentally induced. Much work on the transplantation of tumors induced by various means has followed Fibiger's early reports. The agents employed for induction have included organic compounds, viruses, the milk agent, *Cysticercus fasciolaris*, organ ablation, and hormone administration.

Ellermann and Bang in 1908 (*95*) were the first investigators to observe that filtrable agents are responsible for certain forms of leukemia in chickens. The studies of Rous in 1910 (*245*) on a sarcoma of the fowl stimulated much interest in and subsequent study of the group of spontaneous fowl and rabbit tumors that are not only transplantable but also transmissible by cell-free filtrate (*169, 274*). Starting with the work of Tyzzer in 1907 (*306*), genetic studies also take an important place in the history of transplantable tumors.

From the very beginning, transplantable tumors were recognized as useful tools for cancer research. Studies in which they have been employed include: observations on the pathogenesis and growth of tumors and tumor cells after various routes of transfer in various hosts and in tissue and chick-embryo cultures; analysis of the response of tumors to various physical, chemical, and biologic agents, and the analysis of their content or production of biologic or chemical agents; studies on modes of tumor production, genetics, mutation, the cancer cell, viruses, immunity, and others. The genetics of tumor transplantation has become a highly specialized and significant field of research. In general, the compatibility between a given tumor and its host appears to be genetically determined. Immunological factors can be shown to be active, but it is not presently known how much or how little this is another expression of genetic factors. There have been some studies with the transplantation of normal tissues,

and so far accumulated knowledge has not brought out a distinction between these results and those with transplantable tumors (*212*). For detailed information, and discussions of theories in the field of genetics, useful recent references (*12, 27, 138, 139, 152, 195, 196, 281, 282*) are cited.

Transplantable tumors have been used to study directly much that cannot be studied directly on tumors of man. Although the beginning of chemotherapy as a science goes back to Ehrlich in 1908 (*90*), the use of transplantable tumors for chemotherapeutic trials, or "screening tests," has recently become of prime interest to a large number of workers in cancer research.

In testing therapeutic procedures, the merits of transplantable tumors in comparison with primary tumors should be considered. Generally, the transplanted tumor becomes stabilized after repeated transfers. Frequently in the early generations, tumor growth appears to become more rapid and malignant. During this process the histologic pattern of the tumor may change and become fairly well stabilized. The inbred strain of animal in which the tumor arose may or may not continue to be required for successful transplantation. New potentialities for growth in hosts of genotypes previously resistant have appeared in some tumors, following repeated transplantation. In time, therefore, the behavior and characteristics of growth usually become so firmly established that the tumor is useful for many types of investigation. In the case of many primary tumors, the same uniformity is much more difficult to attain, because the growth rate, killing time, and histologic characteristics may vary considerably and suitable control groups are not always available. However, the anatomic and physiologic environment of primary tumors is not alien, and regression is extremely rare. Studies with both types of tumors may be continued with profit.

It has become apparent that tumors of both animals and man show strong family resemblances; *i.e.*, as a group they behave like tumors or new growths. Transplantable tumors of animals are generally assumed to be analogous to tumors of man. Study of the characteristics of the listed tumors seems to indicate that tumors of different sites of origin in many cases represent different disease entities. Indeed, many of the transplantable tumors have specific individual characteristics, as do certain human tumors, which appear to separate them from all the others. Some transplantable tumors, for example, the leukemias in the mouse, closely resemble acute leukemia in man, both in histologic features and in clinical course. Others, such as mammary tumors of the mouse, do not resemble mammary cancer in human beings. In spite of the wide range of types appearing among transplantable animal tumors, neoplasms representative of such tumors in man as the mixed tumor of the salivary gland, have not yet been found. Species variations are conspicuous among transplantable tumors. The preponderance of mammary carcinomas and leukemias occurring in mice in contrast to rats which develop relatively

few of these tumors, the myoepitheliomas of the salivary glands in mice, the mammary fibroadenomas of rats, and the transmissible virus tumors of rabbits and fowls, represent some of these.

Transformation of one morphologic type of tumor to another during serial transplantation, as a carcinoma to a sarcoma (*7,147,250,282,291*), is a phenomenon that cannot be directly compared with possible similar changes in tumors of human beings. Explanations of this phenomenon which have been proposed are: the morphologic transformation of individual tumor cells or true mutation; the induction by transplanted tumor cells of a new tumor arising from the adjacent tissues of the host; the selection through transplantation or the conditions which it imposes of a specific cell type from a tumor originally of mixed type; selective growth of the stroma of a primary tumor; or, the contamination of a transplanted tumor by another tumor. Proof for assigning any one of these explanations to a particular tumor transformation is lacking, although in some cases the evidence is suggestive for one or another of them. Examples of tumors that have changed morphologic type appear on pages 1314–1315, 1318–1319, 1332, 1334, 1340, 1342, 1348, 1350, 1352, 1356, in the following table.

Collection of the Material

The immediate purpose of the present survey is to make easily available in tabular form the salient practical information concerning the transplantable and transmissible tumors of animals that are now in existence in cancer research laboratories. Most of the data are reported from laboratories in the United States of America. The authors are also indebted to a number of investigators in foreign countries for the inclusion of data which they kindly submitted. Most of the 253 tumors listed are maintained by the 112 investigators who reported them. Their co-operation in furnishing information that could not otherwise have been gathered is deeply appreciated, and is taken as evidence that they expect the survey to be a useful instrument in research. No doubt there are omissions in this first attempt at such a survey. Corrections, more data about tumors already listed, original and review references, and new data about additional tumors are urgently invited.

The need for a survey of transplantable animal tumors was originally suggested by Dr. Dale R. Coman, Dr. Balduin Lucké, and Dr. Milton C. Winternitz. The work of collecting data was sponsored by the Subcommittee on Oncology and the Malignancy Subcommittee of the National Research Council, Washington, D. C., and by the National Cancer Institute, Bethesda, Maryland.

An outline or questionnaire of the information desired was prepared in January of 1951, to be answered by investigators maintaining tumor lines, and the final table was compiled from data submitted by them.

The following valuable data regarding tumors which were submitted

have not been included in the table because it was decided to limit the present paper to continuously transplanted lines in homologous hosts:

1) Tumors transplanted in series to heterologous hosts (H. S. N. Greene). A few references are cited (*55, 73, 143, 242, 278, 303*).

2) Primary tumors arising in inbred strains and studied only intermittently as transplantable tumors. These include spontaneous mammary carcinomas of the mouse (C. P. Leblond, J. H. Williams); spontaneous mammary fibroadenomas in Albany strain rats (A. W. Wright); and rat hepatomas produced by feeding an azo dye (A. Cantero).

3) The transmissible venereal tumor of the dog (F. Bloom, *28*). A rat nephroblastoma (C. T. Olcott, *233*). The tumors are not currently maintained as far as is known.

4) Tumors induced by intraorganal transplantation, but which were not themselves transplanted (G. R. Biskind, *25, 26*).

The table lists only information judged useful for a comparative survey of research on transplantable and transmissible tumors. Original references should be consulted for details on etiology, histology, and biologic behavior of tumors. Data sent by more than one contributor were pooled. In general the authors were faithful to the data submitted. When these were contradictory, they used their best judgment.

Transplantable and transmissible tumors are listed in the following species of animals: mouse, rat, hamster, guinea pig, rabbit, chicken, duck, frog, axolotl, and newt. The data on strains and sublines of mice have been revised, when possible, to conform to a system of designations recommended by *The Committee on Standardized Nomenclature for Inbred Strains of Mice* (*60*).

The tumors were derived from primary tumors, both spontaneous and induced, of specific tissue sites in most cases. Three exceptions, however, were tumors thought to arise from non-neoplastic lesions: the Brown-Pearce rabbit carcinoma from a syphilitic lesion of the scrotum, and a plasmoma and a sarcoma of the rat from *Cysticercus fasciolaris* cysts of the liver.

A description of the histopathology of some transplantable tumors is planned as a fascicle for the "Atlas of Tumor Pathology" under the auspices of the Subcommittee on Oncology.

Acknowledgments

The authors express appreciation to Dr. Isabella Perry, now at the University of California School of Medicine, for valuable help in the initial phases of the preparation of the survey. Grateful acknowledgment is given for the valuable advice of our colleagues at the National Cancer Institute who kindly criticized the manuscript, and in particular to Doctors Thelma B. Dunn, Willard H. Eyestone, Walter E. Heston, and Lloyd W. Law, for extensive scientific and editorial advice.

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Notes on Planning Studies With Transplantable and Transmissible Tumors

There are many technical considerations facing the investigator engaged in work with transplantable tumors.

The tumor host.—The tumors are usually strain-specific and often subline-specific. It may be necessary to establish suitable colonies of inbred animals and maintain accurate breeding records to prevent contamination of the strains by intercrossing. Investigators may choose to induce and transfer tumors in animal colonies already maintained in the institutions with which they are associated. In lieu of this it may be advisable to purchase animals from established genetics laboratories. The F_1 hybrid having one parent of the strain in which the tumor arose is often used as the host for a transplantable tumor (*12*), but it has been shown that the tumor may be changed by such a procedure (*13*). Listed in the table are a few tumors which depend for their continued growth on young hosts, hosts of a particular sex, or castrated or hormone-treated hosts, and some tumors seem to be modified in their growth characteristics by such influences. In addition, the care and health of the animals are important considerations in work with transplantable tumors. Snell (*279*) and Farris and Griffith (*102*) have written useful books on the mouse and on the rat in laboratory investigation.

The tumor.—The maintenance of tumors requires a knowledge of methods and routes of inoculation (*87, 282*), of the size and type of inoculum (*121, 256, 282*), of tranplantation intervals, and of techniques for keeping tumors free of contamination by microbiological agents or by other tumors (*257, 282*). Special techniques are used in transferring some of the transmissible tumors (*48, 245, 272, 273*).

Well recognized changes occur in a tumor after one transfer or repeated transfers, and in different host strains or sublines. Differences in growth characteristics are recognized as a result of varying transfer techniques.

Some developments in experimental techniques.—So-called ascites or "free cell" forms of some solid tumors have been propagated by intraperitoneal transfer of tumors formerly transferred by other routes (*146, 151, 175, 176, 177, 199, 322*). Many although not all transplantable tumors will grow in this form, showing homogeneously distributed cells in the ascitic fluid. This technique makes possible the study of a cell population that approaches a pure culture.

Methods of keeping tumors or tumor grafts in the frozen state (*35, 72, 125, 146, 238, 283, 311*) have been under study by a number of investigators. Different tumors appear to vary in the transplantability which they retain after being subjected to low temperatures for varying lengths of time.

General references.—References to work with transplantable and transmissible tumors of general historical and theoretical interest are the following: *114, 156, 158, 168, 203, 232, 247, 288, 314, 315, 316.*

Index to the Table

An attempt has been made to arrange the neoplastic lesions according to their present tissues of origin, based on the information submitted. There are several exceptions to this general rule dictated by the lack of information available to us in the cases of certain tumors, and by the well known difficulties facing anyone who attempts to set up a satisfactory classification of cancer on any basis.

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I.—Tumors of Epithelial Tissue

I–A.—Tumors presumably of glandular origin with presumptive sites of origin in mammary gland,[1] glandular stomach, salivary gland, liver, pancreas, kidney, adrenal gland, thyroid gland, pituitary gland, ovary, seminal vesicle, sebaceous gland, and harderian gland

Designation and histologic type Name in italics as modified by authors. Synonym in parentheses.	Origin			Host
	Spontaneous (*year if known*)	Induced (*year if known*)	Derivation	Species. Strain and subline when given (*60*).
Adenocarcinoma C3HBA (C3Hba)	1946		Mammary gland	Mouse C3H/An, He, Jax, St, and others.
Adenocarcinoma C3H–HC	1950		Mammary gland	Mouse C3H/Bittner
Adenocarcinoma C3HB	1950		Mammary gland	Mouse C3H/Bittner
Adenocarcinoma H2712	1948		Mammary gland	Mouse C3H/Jax and Hu
Tumor I Indiana U. Adenocarcinoma	1950		Region of mammary gland.	Mouse "C3H/Indiana University"
Adenocarcinoma L1221	1949		Mammary gland	Mouse $C3H_f$/He
Adenocarcinoma MT–8	1948		Mammary gland	Mouse $C3H_f$/He
Adenocarcinoma S(L.E.O.)	1948		Mammary gland	Mouse C3H/An
Adenocarcinoma Ca–Z	1947		Mammary gland	Mouse C3H/An
Adenocarcinoma Ca149	1949		Mammary gland	Mouse C3H

[1] Moulton (*127*) and Dunn (*77*) discuss tumors of the mammary gland in mice.

I.—Tumors of Epithelial Tissue

Growth characteristics				Special features	Contributor(s)	References
Takes (*percent*)	Latent period in days	Kills host in days	Infiltration and metastasis			
100	2–12	14–30	Local invasion. Lung, rare.	Necrosis and hemorrage, minimal. No regression. Explantable to tissue culture. Contains milk agent. Milk agent in host stimulates growth.	4, 13, 18, 23, 40, 106, 108	*13, 14, 15, 16, 20, 144, 312*
100	14	42	Liver and lung.	Hemorrhage.	45	*2, 133.*
100		60	Lymph nodes. Lung.	Hemorrhage. No regression. Explantable.	45	
100	2–3	28			40	
100	7	21	Local invasion.		59	
100	7	14–21		Contains milk agent. Transmission by filtrate, neg.	58	*185, 187*
100	6	15		No milk agent. Transmission by filtrate, neg. [2]	58	*185, 187*
100		30	Local invasion. Lung, late.		88	
100	8–10	28–42	Local invasion. Regional nodes, occasional.	Contains milk agent.	19	
100	5	21	Local invasion. Lung, rare.	Necrosis. No regression.	27, 96	*287*

[2] Although the original tumor and several early passages showed an adenoacanthoma, the tumor has become an adenocarcinoma.

I.—Tumors of Epithelial Tissue—Continued

Designation and histologic type Name in italics as modified by authors. Synonym in parentheses.	Origin			Host
	Spontaneous (*year if known*)	Induced (*year if known*)	Derivation	Species. Strain and subline when given (*60*).
Carcinoma TC3H Mixed cell	1945		Mammary gland, from transplanted adenocarcinoma	Mouse C3H/AnWa
Adenocarcinoma Ca-15	1948		Mammary gland	Mouse C3H/Jax
Adenocarcinoma SPC3H	1950		Mammary gland	Mouse C3H/St
Adenocarcinoma DC4	1949		Mammary gland	Mouse C3H
Tumor #1 Youngner Carcinoma	✓		Mammary gland	Mouse C3H/He
Tumor #2 Youngner Carcinoma	✓		Mammary gland	Mouse C3H/He
Adenocarcinoma E0771 (Eo771)	1939		Mammary gland	Mouse C57BL/6 and 10 C57BL/6,DBA/1
Adenocarcinoma 755 Bagg-Jacksen	1936		Mammary gland	Mouse C57BL/Yale, and others
Adenocarcinoma B1	1949		Mammary gland	Mouse C57BL/6Jax
Adenocarcinoma BW1898	1950		Mammary gland	Mouse C57BL/6Jax
Adenocarcinomas: 7-SBT, 41-SBT, 21-SBT, 49-SBT, 71-SBT	1949		Mammary gland	Mouse C57BL/Dm
Adenocarcinoma C57X		1944 Foster nursing	Mammary gland	Mouse C57BL/Dm

I.—Tumors of Epithelial Tissue—Continued

Growth characteristics				Special features	Contributor(s)	References
Takes (*percent*)	Latent period in days	Kills host in days	Infiltration and metastasis			
100	4–7	14–18	Lung, occasional.	No regression. Explantable to tissue culture and chick embryo. [3]	103	*304*
100	8–10	28–42	Local invasion. Regional nodes, occasional.	Contains milk agent.	19	
100	30–60	30–90	None observed.	No regression. Ascites tumor.	57	*176*
95–100		98–126	None observed.		76, 90	
100	7–14			Explantable to tissue culture.	110	
100	21–28			Explantable to tissue culture.	110	
100	3–5	19–56	Metastasis, rare.	Necrosis, extensive. 11% regression. Explantable. Transmission by filtrate, neg.	40, 52, 100, 104, 106	*54*, *164*, *176*, *221*, *283*, *285*, *300*
100	7–10	42–56	Lung.	Explantable.	53, 72, 104	*9*, *132*, *141*, *165*, *189*
100	2–3	28			40	
100	7–12	80–100			40	
100	7–14			Hemorrhage. Explantable to tissue culture. Transmission by filtrate, neg.	28	
100	7			Hemorrhage. Explantable to tissue culture. Transmission by filtrate, neg.	28	*71*

[3] By passage of tumor cells after several days' sojourn in the subcutaneous tissues of immune hosts, sublines from two types of cells appearing in the original tumor were developed. Subline No. 1 appears to be an anaplastic carcinoma of light-staining cells. Subline No. 2 consists of cords and acini of dark-staining cells showing a tendency to necrosis like that of the same type of cell in the original tumor. Growth characteristics of the sublines resemble those of the original tumor.

I.—Tumors of Epithelial Tissue—Continued

Designation and histologic type Name in italics as modified by authors. Synonym in parentheses.	Origin			Host Species. Strain and subline when given (60).
	Spontaneous (*year if known*)	Induced (*year if known*)	Derivation	
Carcinoma D1905 Heston	1944		Mammary gland	Mouse C57BR/cdSn and Jax
Adenocarcinoma S663	1949		Mammary gland	Mouse DBA/2Jax and Wy
Adenocarcinoma dbaH	1950		Mammary gland	Mouse DBA/Ha
Adenocarcinoma Ca169	1951		Mammary gland	Mouse DBA
Adenocarcinoma Mijono	1950		Mammary gland	Mouse AKR
Adenocarcinoma Ca1	1951		Mammary gland	Mouse CBA
Adenocarcinoma IT$_1$	1949		Mammary gland	Mouse "Swiss/Iowa"
Adenoacanthoma L2132		1949 Milk agent	Mammary gland	Mouse C57BL/He
Ascites tumor Ehrlich	As carcinoma		Mammary gland, from transplanted carcinoma	Mouse Not restricted to a particular strain
Carcinoma 63 Bashford (English)	1907		Mammary gland	Mouse Not restricted to a particular strain

I.—Tumors of Epithelial Tissue—Continued

Growth characteristics				Special features	Contributor(s)	References
Takes (*percent*)	Latent period in days	Kills host in days	Infiltration and metastasis			
100	7	35–42		No milk agent.	93	
100	10–14	28–35			93	
100	16–18	60	Lymph nodes.	No regression. Explantable.	45	
100	14	42	Local invasion. Lung, rare.	No regression.	27, 96	
100	4	63–77	Local invasion. Lung, occasional.	Necrosis, extensive. 10% regression. Explantable. Transmission by filtrate, neg. Anaplastic.	100	
70	7–9	14–18	Local invasion.	Regression, rare.	27, 96	
80		40–60		Explantable to chick embryo. Transmission by filtrate, neg.	49	
100	5–7	20	None observed.	Contains milk agent. [4]	58	
100	4–6	14–28	Solid form: lung. Ascites form: local invasion.	Necrosis, extensive (solid form). 1% regression. Explantable. Transmission by filtrate, neg. Undifferentiated. Ascites tumor. [5]	2, 50, 57, 83 100	*146, 151, 174, 176, 199, 242, 299*
95	4	28–42	Lung, occasional.	Necrosis, extensive. 4% regression. Undifferentiated. Explantable.	100	*18, 52, 54, 130, 300*

[4] The mixed epithelial tissue tumor has retained its original histologic appearance. (Contrast with *Adenocarcinoma MT-8*, pages 1306–1307.)

[5] The ascites tumor was derived from the solid tumor in 1932 (*199*). According to K. Bayreuther ("Der Chromosomenbestand des Ehrlich-Ascites-Tumors der Maus," Ztschr. f. Naturforsch. 7b: 554–557, 1952), the Ehrlich carcinoma arose in 1896. Three strains of the ascites form of the tumor are referred to.

I.—Tumors of Epithelial Tissue—Continued

Designation and histologic type Name in italics as modified by authors. Synonym in parentheses.	Origin			Host Species. Strain and subline when given (60).
	Spontaneous (*year if known*)	Induced (*year if known*)	Derivation	
Adenocarcinoma dbrB (dBrB)	1918		Mammary gland	Mouse DBA/1
Adenocarcinoma RC	1943		Mammary gland	Mouse DBA
Adenocarcinoma DC2	1949		Mammary gland	Mouse MA
Adenocarcinoma DC3	1948		Mammary gland	Mouse BALB
Carcinoma S674	1950		Mammary gland	Mouse RIII/Sn and Wy (will not grow in RIII/Jax)
Adenocarcinoma SPAH	1950		Mammary gland	Mouse A/St
Carcinoma 15091a	1928		Mammary gland, from transplanted adenocarcinoma	Mouse A/Jax and Ha
Adenocarcinoma TA3	✓		Mammary gland	Mouse A/HeHa
Adenocarcinoma R2426	1939		Mammary gland	Rat August/7322
Adenocarcinoma R2857	1948		Mammary gland	Rat August/7322
Adenocarcinoma IRC 741		1950 2-Acetylaminofluorene	Mammary gland	Rat Fischer/344

I.—Tumors of Epithelial Tissue—Continued

Growth characteristics				Special features	Contributor(s)	References
Takes (*percent*)	Latent period in days	Kills host in days	Infiltration and metastasis			
100	4–5	21–24	Liver and lung.	Explantable to tissue culture.	40, 45	*2, 133, 283, 285, 292*
100				Ulceration, at 14 days.	104	
65–70		70–91	None observed.		76, 90	
95–100		63–77	None observed.		76, 90	
100	10	28–35			93	
100	30–45	45–75		No regression.	57	*176*
100	2–3	Solid form: 22–26 Ascites form: 7–10		Hemorrhage. No regression. Anaplastic. Ascites tumor.	40, 57	*138, 163, 164, 176, 280, 283, 284, 285*
100	2–3	Solid form: 15–22 Ascites form: 6–10		No regression. Undifferentiated. Ascites tumor.	50, 57	*151, 176,*
100	14	40–90	Local invasion. Lung, late.	No regression. Explantable to tissue culture. Transmission by filtrate, neg. Female hormone excites secretion.	31, 45	*50, 81, 93*
100	10	30–35	Local invasion. Lymph nodes. Lung.	No regression.	31	
100	10	40–45	Local invasion.	No regression.	31	

I.—Tumors of Epithelial Tissue—Continued

Designation and histologic type Name in italics as modified by authors. Synonym in parentheses.	Origin			Host Species. Strain and subline when given (*60*).
	Spontaneous (*year if known*)	Induced (*year if known*)	Derivation	
Adenocarcinoma Webster		✓ 2-Acetylaminofluorene	Mammary gland	Rat Hooded/R.V.H.
Adenocarcinoma, Shay "Duct cell type"		1946 20-Methylcholanthrene	Mammary gland	Rat Wistar (random bred)
Carcinoma #10 Lewis		1950 20-Methylcholanthrene	Mammary gland	Rat Lewis, inbred
Carcinoma #303		1949 20-Methylcholanthrene	Glandular stomach	Mouse C3H/N
Carcinoma #328		1949 20-Methylcholanthrene	Glandular stomach	Mouse C57BL/N
Carcinoma #342		1950 20-Methylcholanthrene	Glandular stomach	Rat M520 (Marshall)
Myoepithelioma 243C	1950		Salivary gland	Mouse BALB/c
Pleomorphic carcinoma BW1081	1949		Salivary gland	Mouse BALB/cHu
Submaxillary tumor C-CBA	1950		Submaxillary gland	Mouse (CBA × BALB/c)F_1
Hepatoma #10 Andervont		1946 *o*-Aminoazotoluene	Liver	Mouse BALB/cJax
Hepatoma C954	1940		Liver	Mouse C57L

I.—Tumors of Epithelial Tissue—Continued

Growth characteristics				Special features	Contributor(s)	References
Takes (*percent*)	Latent period in days	Kills host in days	Infiltration and metastasis			
25	11	60	Local invasion. Regional nodes. Lung.		105	
80–100 In female babies		90–180	Local invasion.	No regression.	84	*263*
100		21–28	Local invasion. No metastasis observed.		61	
95	8–14	90–100	Lymph nodes. Lung.	No regression.	97	*290*
95	5–7	90–100	Lung.	No regression. Hypervolemia.	97	*290*
95	10–15	90		No regression.	97	*150, 290*
100	7–14	90–120	Local invasion. Lung, rare.	Necrosis. Leukemoid reaction. [6]	30	*19*
100	4–6	70–84		[6]	40	
100	21	60–90	None observed.	Leukemoid reaction. [6]	56	
93	14	91–182	None observed.	No regression. Transmission by filtrate, neg. [7]	100	*6, 300*
100	2–3	22–24	None observed.	Ascites tumor.	40, 57	*58, 176, 190, 285*

[6] These three tumors, and similar ones arising from the salivary glands in mice, do not have a distinctively glandular appearance. Composed of "myoepithelial" cells, they are sometimes referred to as "Grady salivary gland tumor." A milky or mucous secretion is described. Number 243C has been discontinued.

[7] A hemangioendothelioma developed at the site of transplantation at the National Cancer Institute. In Sugiura's laboratory, however, at the Sloan-Kettering Institute, the tumor continues as a hepatoma.

I.—Tumors of Epithelial Tissue—Continued

Designation and histologic type Name in italics as modified by authors. Synonym in parentheses.	Origin			Host
	Spontaneous (*year if known*)	Induced (*year if known*)	Derivation	Species. Strain and subline when given (*60*).
Hepatoma 98/15	1940		Liver	Mouse C3H Not subline specific
Hepatoma 112/B		1945 Chloroform	Liver	Mouse A/He
Hepatoma LC 18		1947 2–Acetyl-aminofluorene	Liver	Rat Fischer/344
Hepatoma #3683		1951 2-Diacetyl-aminofluorene	Liver	Rat A × C/9935 (Irish)
Hepatoma N (Hepatoma NK Novikoff)		1951 *p*-Dimethyl-aminoazobenzene (Butter yellow)	Liver	Rat Sprague-Dawley-Holtzman
Hepatoma #3924A		1951 2-Diacetyl-aminofluorene and hormones	Liver	Rat A × C/9935 (Irish)
Hepatoma #3924C Cholangioma		1951 2-Diacetyl-amino fluorene	Liver	Rat A × C/9935 (Irish)
Hepatoma #3930		1951 2-Diacetyl-aminofluorene	Liver	Rat A × C/9935 (Irish)
Pancreatic carcinoma SB4	1948		Pancreas	Rat "Curtis-Dunning/Donner"
Kidney carcinoma Lucké Adenocarcinoma	1932 In nature		Kidney	Leopard frog (*Rana pipiens*)

I.—Tumors of Epithelial Tissue—Continued

Growth characteristics				Special features	Contributor(s)	References
Takes (*percent*)	Latent period in days	Kills host in days	Infiltration and metastasis			
75 (100, in young hosts)	17–21 (14, in young hosts)	120–180	None observed.	Necrosis and ulceration.	26, 38	*6, 176*
75	17–21	120–180	None observed.	Ulceration. [8]	38	*101*
100	14	45–50	Local invasion. Lymph nodes. Lung.	Regression, after ulceration. Explantable to tissue culture.	31	*80*
100	10–14	14–28	Local invasion.	Hemorrhage.	71	*211*
100 By intraperitoneal transplantation	3–10	7–20	Local invasion.	Necrosis, minimal.	47, 68	*211*
100	10–14	25–30	Lung.		71	
100	10–14	20–25			71	*211*
100	10–14	21–28			71	
100	10–14	30		Undifferentiated.	52	*260*
10–100 Influenced by site of inoculation			Local invasion. Liver, lung, and other sites.	Explantable to tissue culture. Transmission by desiccated material. Benign and malignant forms. Virus postulated.	64	*169, 202, 203, 204*

[8] The tumor has been discontinued.

I.—Tumors of Epithelial Tissue—Continued

Designation and histologic type Name in italics as modified by authors. Synonym in parentheses.	Origin			Host Species. Strain and subline when given (60).
	Spontaneous (*year if known*)	Induced (*year if known*)	Derivation	
Adrenal cortical tumor Illinois		1950 20-Methylcholanthrene and estrogen	Adrenal gland	Mouse BALB/c
Adrenal cortical carcinoma W35		1950 Castration	Adrenal gland (cortex)	Mouse CE/Wy
Adrenal cortical carcinoma W92		1950 Castration	Adrenal gland (cortex)	Mouse CE/Wy
Adrenal tumor Lorenz		1951 X radiation and castration	Adrenal gland (cortex?)	Mouse $C3H_1$/He A/He
Thyroid tumor #180 Morris Colloid adenoma		1946 Thiouracil	Thyroid gland	Mouse C3H/An
Chromophobe pituitary tumor Furth (1) Dependent on absence of thyroid gland		1950 Thyroid gland destroyed by I^{131}	Pituitary gland	Mouse C57BL/Rl
Chromophobe pituitary tumor Furth (2) Independent of thyroid gland	1951		Pituitary gland, from transplanted dependent tumor	Mouse C57BL/Rl
Ovarian tumor Symeonidis Mucinous adenocarcinoma	1949 (10)		Ovary	Rat Osborne-Mendel
Carcinoma Flexner-Jobling	1906		Seminal vesicle	Rat Not restricted to a particular strain
Hamartoma malignum BS_1	1949		Gluteal region (sebaceous gland?)	Rat "Albino"

[10] The mother of the rat bearing the original tumor was treated during pregnancy with 2-acetylaminofluorene and progesterone.

I.—Tumors of Epithelial Tissue—Continued

Growth characteristics				Special features	Contributor(s)	References
Takes (*percent*)	Latent period in days	Kills host in days	Infiltration and metastasis			
90	9	70	None observed.	Necrosis. No regression.	56	
100	60	"a long time"		Hormonally active.	108	*320*
62	60	"a long time"			108	*320*
100	7	60	Metastasis, rare.	Hormonally inactive.	21, 62, 63	
90	21–42	140		[9]	71	*224, 225, 317*
100 In the absence of thyroid gland	90–120		Regional nodes.	No regression. Hormonally active: FSH, LSH, TSH and bile-duct stimulating hormone.	43	*118, 119, 123, 124, 136*
100	45–90		Lymph nodes.	Hormonally active: FSH, LSH, and TSH.	43	*118, 123, 124*
88–100	14	Solid form: 60 Ascites form: 40	Lymph nodes. Lung and liver.	Ascites tumor. Organotropy, ovary.	102	*301*
100	4–5	17–70	Local invasion. Lung, occasional.	Necrosis, extensive. 25% regression. Undifferentiated. Explantable. Ascites tumor. [11]	80, 100	*49, 110, 111, 179, 191, 254, 297, 298, 300, 318*
90				Necrosis. Hemorrhage.	7	

[9] The tumor, once dependent on thiouracil, is now autonomous. It takes up iodine and forms thyroxine.

[11] From the evidence from the literature the original tumor was a "polymorphous sarcoma with some glandular elements" which during serial transplantations underwent successive changes to alveolar carcinoma, to adenocarcinoma, to undifferentiated carcinoma.

I.—Tumors of Epithelial Tissue—Continued

Designation and histologic type Name in italics as modified by authors. Synonym in parentheses.	Origin			Host Species. Strain and subline when given (60).
	Spontaneous (*year if known*)	Induced (*year if known*)	Derivation	
Adenocarcinoma JS2	1947			Rat "Curtis-Dunning/Donner"
Harderian gland carcinoma 2226		1945 2-Diacetylaminofluorene	Posterior region of orbital cavity (harderian gland?)	Rat Buffalo, and Marshall
I–B.—Tumors of a postulated but unconfirmed glandular origin. These are pulmonary, ovarian granulosa-cell and lutein-cell, and testicular interstitial-cell tumors				
Pulmonary adenocarcinoma C4461		1943 20-Methylcholanthrene	Lung	Mouse A/He
Carcinoma MT8	✓		Lung?	Mouse Not restricted to a particular strain
Pulmonary adenoma Cohen		1947 Urethane	Lung	Mouse C3H
Granulosa-cell tumor Eschenbrenner		1947 X radiation	Ovary	Mouse $C3H_f$/He
Ovarian tumor E4478 Granulosa-cell type	1946		Ovary	Mouse C3H/An $C3H_f$/He
Granulosa-cell tumor XIV		✓ X radiation	Ovary	Mouse RF × AKR hybrids
Ovarian tumor OL Granulosa-cell type		1947 Intrasplenic ovarian graft, in castrate host	Ovary	Mouse A/St

I.—Tumors of Epithelial Tissue—Continued

Growth characteristics				Special features	Contributor(s)	References
Takes (*percent*)	Latent period in days	Kills host in days	Infiltration and metastasis			
100	5	21		Necrosis. Undifferentiated.	52	*260*
100	7–10	28	Local invasion. Lung.	Necrosis, minimal.	71	*210, 226 244*
100	7	42	None observed.	[12]	51	*4, 289*
90	8–14	60–120	Local invasion.	Necrosis. Ulceration. "Unorganized arrangement."	92	*189, 278*
100	6–7	14–28	Local invasion.	Undifferentiated.	19	
100	14	42	Local invasion. Lung, rare.	Necrosis. Hormonally active. Hypervolemia. [13]	38, 62	*200*
50–75	120–240	240–360	Local invasion. No metastasis observed.	Hormonally inactive.	30	
50–75	30–60	30–60	None observed.	No regression. Hormonally active. Hypervolemia.	43	*10*
100	10	60–90	Local invasion. Lung, liver, adrenal, spine, and other sites.	Hormonally inactive. [14]	53	*194*

[12] The tumor has remained an adenocarcinoma in contrast to the usual change in histologic pattern of transplanted pulmonary adenocarcinomas (*291*).

[13] There may be an association between the primary tumor and the development of mammary sarcomas. The transplantable tumor has been discontinued.

[14] The tumor, now independent, grew at first only in gonadectomized female hosts.

I.—Tumors of Epithelial Tissue—Continued

Designation and histologic type Name in italics as modified by authors. Synonym in parentheses.	Origin			Host Species. Strain and subline when given (*60*).
	Spontaneous (*year if known*)	Induced (*year if known*)	Derivation	
Ovarian tumor IX Lutein-cell type		✓ X radiation	Ovary	Mouse RF × AKR hybrids
Testicular tumor 3AC$_1$SS Interstitial-cell type		1942 Estradiol benzoate	Testis	Mouse C3H (A × C3H)F$_1$ (C3H × A)F$_1$ (C3H$_f$ × A)F$_1$
Testicular tumor 20AB$_2$T Interstitial-cell type		1943 Estradiol benzoate	Testis	Mouse (A × CBA)F$_1$ (A × C3H)F$_1$ (C57BL × CBA)F$_1$
Testicular tumor Bonser Interstitial-cell type		1944 Triphenyl-ethylene	Testis	Mouse A/StBr
Leydig cell tumor Furth	✓ Exposed to X radiation.		Testis	Mouse RF/Fu
I-C.—Tumors of nonglandular epithelial origin, with presumptive sites of origin in the skin,[18] the forestomach, and the uterine cervix				
Tumor #1 Cowdry (Tumor I) Squamous-cell type		1944 20-Methyl-cholan-threne	Skin	Mouse "Swiss"
Tumor #D Cowdry Squamous-cell type	1950		Skin	Mouse C57L
Carcinoma G8755 Squamous-cell type	1949		Forestomach	Mouse C3H$_f$/He
Carcinoma #338 Squamous-cell type		1950 20-Methyl-cholan-threne	Glandular stomach	Rat M520 (Marshall)
Gastric carcinoma line A Squamous-cell type		20-Methyl-cholan-threne	Fetal stomach	Mouse A/Jax

[18] Carruthers (*51*) reviews the chemistry of epidermal tumors in mice.

I.—Tumors of Epithelial Tissue—Continued

Growth characteristics				Special features	Contributor(s)	References
Takes (*percent*)	Latent period in days	Kills host in days	Infiltration and metastasis			
50–75	20–80	30–90	Liver, from splenic grafts.	No regression. Hormonally active. Polycythemic hypervolemia.	43	*10, 122* **140**
100	10	21–90	Lung, late.	Hemorrhage. [15]	44, 108	*108, 126*
100	10	28–90	Lung.	Hormonally active. Hypervolemia, occasional. [15]	44	*126*
90			Regional nodes. Lung.	Hemorrhage.	6	*29, 30, 31*
10–20	30–65	120–240	None observed.	No regression. Hormonally active.	43	
30–70		60–75	Local invasion. Metastasis, rare.	Necrosis. Explantable to chick embryo.	24	*61*
100		"Slow"	None observed.	Necrosis.	24	
100	21–42	120–150	Local invasion. Lung.	Necrosis.	51	
25–75		60–75		Necrosis. [16a]	97	
90–100	5–14	90–180	None observed.	Necrosis. Hemorrhage. Regression, after ulceration.	14, 77	

[16] The tumor, now independent, grew at first only in hosts given estrogen pellets.
[16a] The tumor has been discontinued.

I.—Tumors of Epithelial Tissue—Continued

Designation and histologic type Name in italics as modified by authors. Synonym in parentheses.	Origin			Host Species. Strain and subline when given (60).
	Spontaneous (*year if known*)	Induced (*year if known*)	Derivation	
Cervical carcinoma T145 Squamous-cell type		1951 20-Methylcholanthrene	Cervix	Mouse C3H/An
Carcinoma 1025 Furth		1945 20-Methylcholanthrene	Cutaneous or subcutaneous tissue	Mouse AKR
Carcinoma DC5	1950		Skin	Mouse "ND"
Carcinoma Krebs 2	1933		Inguinal region (male host)	Mouse Not restricted to a particular strain
Papilloma Shope Squamous-cell type	✓ In nature		Skin	Rabbit Cottontail and domestic (*Oryctolagus*)
Carcinoma Vx2 (V2)	1938		Skin, by way of *Papilloma Shope*	Rabbit Not restricted to a particular strain
Carcinoma Brown-Pearce (Epithelioma Brown-Pearce)	1921 In host with experimental syphilis		Syphilitic lesion of the scrotum	Rabbit Not restricted to a particular strain
Carcinoma HC1 Basal-cell type	1949		Eyelid	Syrian hamster

I.—Tumors of Epithelial Tissue—Continued

Growth characteristics				Special features	Contributor(s)	References
Takes (*percent*)	Latent period in days	Kills host in days	Infiltration and metastasis			
100	7	60–120	Metastasis, rare.	Necrosis. No regression.	72	
100	4	28–49	Local invasion. Metastasis, rare.	No regression. Undifferentiated. Explantable.	100	*300*
95–100		49–84	None observed.	Necrosis. Anaplastic.	76, 90	
100	Solid form: 14–21 Ascites form: 4–10	Solid form: 30 or more. Ascites form: 5–30	Local invasion. Metastasis observed.	Anaplastic. Transmission by filtrate, neg. Ascites tumor (1950).	11, 50, 57, 78	*151, 176, 177, 310*
100	6–18		None observed.	Regression, of intracutaneous implants. Not explantable. Transmission by filtrate, pos. Virus tumor. [17]	89	*40, 109, 169, 178, 248, 272,*
84–100	10–21	42–120	Local invasion. Regional nodes. Lung.	Necrosis. Anaplastic. Explantable to tissue culture. Transmission by filtrate, neg. [17]	20, 42, 54, 59, 79	*131, 169, 170, 205, 249, 313*
75–100	4–7	10–100	Local invasion. Liver, adrenal, and other sites.	Regression, of intracutaneous implants. Anaplastic. Explantable, to chick embryo. Transmission by filtrate, neg.	17, 20, 73, 81	*8, 37, 38, 52, 53, 59, 94, 112, 240, 255*
100		70–100	Lymph nodes. Lung.		25	

[17] The papilloma is a benign tumor and does not kill, although secondary malignant alteration in the virus-infected cells of the papilloma is frequent. The *Carcinoma Vx2* is a transplantable tumor derived from a primary tumor which was the result of this transformation.

II.—Lymphomas and Leukemias [18, 19]

II–A.—Lymphosarcoma or lymphocytic leukemia, or both, with neoplastic involvement of organs and tissues, or leukemic blood, or both, in most instances

Designation and histologic type Name in italics as modified by authors. Synonym in parentheses.	Origin			Host
	Spontaneous (*year if known*)	Induced (*year if known*)	Derivation	Species. Strain and subline when given (*60*).
Lymphatic leukemia L4616	1951		Spleen and lymph node.	Mouse AKR/Lw
Lymphatic leukemia Leu 3	1950		Thymus gland	Mouse AKR/M
Lymphatic leukemia Ak	1948		"Leukemic tissues"	Mouse AK/n C3H newborn C57BL newborn
Lymphoid leukemia Ak4			Spleen	Mouse AKR/M and Sk
Lymphoid leukemias Furth	✓	✓ X radiation	"Leukemic tissues"	Mouse AKR/Fu RF/Fu
Lymphoid leukemia VII	1950		Thymus gland	Mouse AKR
Lymphosarcoma 1527	1950		Spleen	Mouse AKR/M
Lymphosarcoma Patterson	1947		Region of thymus gland	Mouse AKR/M
Lymphosarcoma Mecca	1949		Region of mammary gland, from transplanted carcinoma	Mouse AKR/M
Lymphosarcoma 6C3HED Gardner		1941 Estradiol benzoate	Thymus gland	Mouse C3H, CBA, CHI

[18] Engelbreth-Holm (*100*), Kirschbaum (*172*), Dunn (*75*) and Furth (*117*) review leukemia in animals.

[19] Established lines of transplantable leukemia show considerable variations in 1) the extent of the local growth that precedes the onset of leukemic changes in the blood, and 2) the organs and tissues affected by leukemic infiltration. Neoplastic spread may depend in part upon the size of the inoculum (*121*), and also upon the route of transfer. Subcutaneous inoculation results in tumor growth which may remain local for a relatively long time before generalized dissemination occurs, while dissemination is often rapid and widespread following intravenous, intraperitoneal, or intrasplenic inoculation. A transplanted neoplasm that apparently does not become disseminated is listed on page 1330.

II.—Lymphomas and Leukemias

Growth characteristics				Special features	Contributor(s)	References
Takes (*percent*)	Latent period in days	Kills host in days	Infiltration and metastasis			
100	5–7	10	Widely disseminated.		58	
100	5–7	10–14	Widely disseminated.	No regression.	27, 96	*68*
100	10–15	14–21	Widely disseminated.		19	*145*
99	5	7–12	Widely disseminated.		48, 91	*275*, *276*, *277*
100			Widely disseminated.		43	
75	14	21	Widely disseminated.		65, 98	
100		19	Widely disseminated.		37	
100	4	14–28	Widely disseminated.	Necrosis, minimal. 1% regression. Explantable. Transmission by filtrate, neg.	100	*176*, *300*
100	4	14–21	Widely disseminated.	Necrosis, minimal. 1% regression. Explantable. Transmission by filtrate, neg.	100	*300*
100	2–7	Solid form: 14–24 Ascites form: 7–10	Widely disseminated.	Necrosis, minimal. Transmission by filtrate, neg. Ascites tumor.	40, 44, 50, 56, 57, 80, 108	*127*, *151*, *176*

II.—Lymphomas and Leukemias—Continued

Designation and histologic type Name in italics as modified by authors. Synonym in parentheses.	Origin			Host Species. Strain and subline when given (60).
	Spontaneous (*year if known*)	Induced (*year if known*)	Derivation	
Lymphatic leukemia 926F	1948		Spleen	Mouse F/StIll; and hybrids
Lymphatic leukemia 1016F	1949		Thymus gland	Mouse F/StIll
Lymphoid leukemias: 868, 876, 926R	1948		Spleen	Mouse F/StIll
Lymphoid leukemia 100	1948		Spleen	Mouse F/StIll
Lymphatic leukemia P1534	1940		Thymus gland	Mouse DBA/2
Lymphoid leukemia L1210		1948 20-Methylcholanthrene	Spleen and lymph node	Mouse DBA/2
Thymoma Dalton (Thymoma dba) Lymphosarcoma	1947		Thymus gland	Mouse DBA/2
Lymphosarcoma DS9	1950		Region of pancreas	Mouse DBA
Lymphosarcoma C43 Kaplan		1947 X radiation	Thymus gland	Mouse C57BL/St and He
Lymphosarcoma TT15		1949 X radiation		Mouse C57BL
Leukemia Line I MacDowell Lymphocytic	1929		Spleen	Mouse C58/M
Lymphoid leukemia L3054	1949		Spleen and lymph node	Mouse C58/Lw
Lymphoid leukemia HE8186	1949 Nitrogen mustard treated		Spleen and lymph node	Mouse A/He

II.—Lymphomas and Leukemias—Continued

Growth characteristics				Special features	Contributor(s)	References
Takes (*percent*)	Latent period in days	Kills host in days	Infiltration and metastasis			
100	7	8–12	Widely disseminated.	No necrosis. No regression.	56	
100	7–10	14	Widely disseminated.	Necrosis, minimal. No regression.	56	
100	4–13	7–16	Widely disseminated.	926R, resistant to folic acid antagonists.	56	*173*
100	20	45	Widely disseminated.		56	*173*
100	3–5	10–12		Hemorrhage.	40	*182, 283, 284, 285*
100	2–3	8–9	Widely disseminated.	[20]	27, 58, 66, 96	*144, 183, 184, 186 188*
Solid form: 70 Ascites form: 90–100	3–4	Solid form: 13–16 Ascites form: 10–14	Widely disseminated.	Ascites tumor.	41, 48, 50, 57	*134, 151, 176*
100		28–42		No necrosis. No regression.	76, 90	
100	7–10	30	Widely disseminated.		53, 88	
	7–10	60			9	
100		4	Widely disseminated.		58	*207, 208, 243, 302*
100	6	12	Widely disseminated.	Necrosis, minimal. [21]	58	
100	6–8	14	Widely disseminated.	Necrosis, minimal.	58	

[20] This line is sentitive to folic-acid antagonists. There are also four sublines, two of these dependent (on A-methopterin and 8-Azaguanine respectively), and two resistant to each of these two compounds.

[21] The tumor is sensitive to folic-acid antagonists. A subline is resistant to adenopterin.

II.—Lymphomas and Leukemias—Continued

Designation and histologic type Name in italics as modified by authors. Synonym in parentheses.	Origin			Host Species. Strain and subline when given (60).
	Spontaneous (*year if known*)	Induced (*year if known*)	Derivation	
Lymphoma #2 (L#2) Lymphocytic		1946 X radiation	Lymph node	Mouse A (BALB/c × A)F_1/ Bittner and He
Lymphoid sarcoma 15BL	1946		Mediastinum	Mouse C3H, BL, CBA, and hybrids thereof
Leukemia L3660 Lymphocytic	1951		Spleen and lymph node	Mouse BALB/c
Lymphosarcomas: TT8 and TT10		1949 X radiation		Mouse CF1 (Carworth)
Lymphosarcoma Krebs, Rask-Nielsen, Wagner		1928 X radiation	Mediastinum	Mouse Not restricted to a particular strain
Lymphatic leukemia Shay		1948 20-Methyl-cholan-threne	Spleen	Rat Wistar (random bred), Sherman, Long-Evans
Lymphosarcoma Murphy-Sturm		1938 1,2,5,6-Di-benzan-thracene	Lymph node and blood	Rat Wistar, Carworth, Sprague-Dawley
Lymphoma A40 Lymphocytic	1948		Lymph node	Guinea Pig Strain 2 (Family 2)
Lymphoma #1 (L–1) (L#1) Lymphosarcoma		1946 X radiation	Mediastinum	Mouse (BALB/c × A)F_1/ Bittner and He A/He,St,Jax, Bittner "ABC" DBA/2

II.—Lymphomas and Leukemias—Continued

Growth characteristics				Special features	Contributor(s)	References
Takes (*percent*)	Latent period in days	Kills host in days	Infiltration and metastasis			
100	7	9–24	Widely disseminated.	Necrosis, minimal. Hemorrhage. No regression. Ascites tumor.	62, 86	*144, 266*
99	7	14–21	Widely disseminated.		44	
100	30	30–45	Local invasion. Regional nodes.	Large tumor growth.	58	
	7–10	60			9	
65–70	4–8	Solid form: 14–28 Ascites form: 8–16	Widely disseminated.	Necrosis, minimal. 30–35% regression (A mice). Ascites tumor.	57	*176, 180*
100 In babies	5–6	10–14	Widely disseminated.	No regression.	84	*262, 264, 265*
75–100	4–8	8–35	Widely disseminated.	Necrosis, minimal. Hemorrhage. 20% regression. Explantable to tissue culture. Transmission by filtrate, neg.	69, 83, 95, 99, 100	*228, 229 293*
90	28–42	150	Widely disseminated.		62	
100	7	24–40	Local invasion. Regional nodes. (22)	Necrosis, minimal.	62, 82, 86, 88	*20, 144, 266*

[22] The tumor appears to be a lymphosarcoma without leukemic blood (contrast with *Lymphoma #2*, page 1330). There is local invasion and (late) embolic spread, but the blood does not become leukemic and there are no true metastases. The cells of L#1 are approximately twice the size of those of L#2 and appear to have a tetraploid number of chromosomes in the nucleus. L#2 is rich in alkaline phosphatase while L#1 contains no detectable amount.

II.—Lymphomas and Leukemias—Continued

II–B.—Myelocytic leukemia [22] *(including chloroleukemia)*

Designation and histologic type Name in italics as modified by authors. Synonym in parentheses.	Origin			Host
	Spontaneous (*year if known*)	Induced (*year if known*)	Derivation	Species. Strain and subline when given (*60*).
Myeloid leukemia 15F	1948		Spleen	Mouse F/StIll; and hybrids FA/Ki
Myeloid leukemias: X, 274, 686, 765	1948		Spleen	Mouse F/StIll (#274, in FA)
Myeloid leukemia 15A	1948		Spleen	Mouse F/StIll
Myeloid leukemia C1498	1941		Liver and spleen	Mouse C57BL/6
Myelogenous leukemia Shay Chloroma type		1949 20-Methylcholanthrene	Blood	Rat Wistar (random bred)

II–C.—Reticulum-cell sarcoma

Reticulum-cell sarcoma F8469	1948		Wall of vagina and ovaries	Mouse (C57L × A)F_1
Reticulum-cell sarcoma 48814	1951		Liver	Mouse C57BL × DBA
B-leukemia Bichel Reticulum-cell type			"Thymus gland?"	Mouse "Mixed stock"
Sarcoma R39 Reticulum-cell type	1916		Region of groin, from transplanted fibroadenoma to fibrosarcoma	Rat Not restricted to a particular strain

[22] Barnes and Sisman (*11*) review myelocytic leukemia in mice.

II.—Lymphomas and Leukemias—Continued

Growth characteristics				Special features	Contributor(s)	References
Takes (*percent*)	Latent period in days	Kills host in days	Infiltration and metastasis			
100	12	15–20	Widely disseminated.	No necrosis. No regression. Transmission by filtrate, neg.	56	*128, 173*
100	5–15	8–18	Widely disseminated.		56	*128, 173*
100		30–60	Widely disseminated.		56	
100	3–8	10–16	Widely disseminated.	No regression. [24]	27, 40, 52, 96	*68, 182, 283, 284, 285*
80–100 In babies	42–70	60–90	Widely disseminated.		84	*264*
50–80	120–300	120–450	Lymph nodes. Liver, ovaries.	Regression, local. Organotropy, liver. [25]	30	*58, 76, 137*
90–100	14–21	60–90	Liver.	Regression, local. Organotropy, liver. [25]	30	
80–90			Widely disseminated.	15–20% regression. Expantable to tissue culture. Organotropy. [25]	5	*22*
100	4	21–56	Local invasion. Dissemination, rare.	Necrosis, extensive. 4% regression. Explantable.	100, 101	*255, 300*

[24] The tumor is histologically not a typical myelocytic leukemia at the present time.

[25] These frequently occurring tumors in mice have also been called "malignant hepatic histiocytoma" (*137*), "a transferable liver neoplasm" (*56*), "a transmissible monocytoma" (*192*), "reticuloendotheliosis" (*214*), "a neoplasm of monocytes" (*116*), and "monocytic leukemia or monocytosis" (*120*).

II.—Lymphomas and Leukemias—Continued

Designation and histologic type Name in italics as modified by authors. Synonym in parentheses.	Origin			Host Species. Strain and subline when given (60).
	Spontaneous (*year if known*)	Induced (*year if known*)	Derivation	
Lymphosarcoma R2788 Reticulum-cell type	1947		Mesenteric lymph nodes	Rat A × C/9935 (Irish)
Lymphosarcoma Bagg Reticulum-cell type				Rat Not restricted to a particular strain
Reticuloendothelioma #9	1943		Mesenteric lymph nodes	Rat Wistar
Reticuloendothelioma #19	1950		Mesenteric lymph nodes	Rat King A, inbred
Reticulum-cell-like tumor, Copeland		1948 2-Acetylaminofluorene	Mammary gland, from transplanted carcinoma to sarcoma	Rat "A.E.S."
Reticulum-cell-like sarcoma, Yoshida		1943 *o*-Aminoazotoluene and potassium arsenite	Chylous ascitic fluid	Rat "Albino Japanese" hybrid
II-D.—Plasma-cell tumor				
Plasma cell leukemia Bichel	1950		Region of axilla	Mouse AK/a
Plasmoma IRS 6820		1936 *Cysticercus fasciolaris*	Cyst of liver	Rat A × C/9935 (Irish)

II.—Lymphomas and Leukemias—Continued

Growth characteristics				Special features	Contributor(s)	References
Takes (*percent*)	Latent period in days	Kills host in days	Infiltration and metastasis			
100	8–10	30	Local invasion. Lymphoid tissues. Lung.	No regression.	31	*66*
100	7	28	Local invasion. Dissemination, rare.	Necrosis, minimal. Hemorrhage. High percentage of regression.	83	
80		14–21	Widely disseminated.		61	*103, 160*
65		30–60	Local invasion. Lymph nodes.		61	
90–100			Local invasion. Lung.	[26]	22	*62, 99*
91–100 (50, single cell transplant)	Ascites form: 3–4	Ascites form: 6–14	Local invasion. Dissemination, late.	Regression observed. Explantable to chick embryo. Transmission by filtrate, neg. Ascites tumor.	16, 55, 57, 109	*176, 321, 322, 323*
100	6–8		Widely disseminated.	Necrosis. No regression. Explantable to tissue culture. Ascites tumor.	5	*21*
100	10	35–40	Local invasion. Lymph nodes. Lung.	No regression. Explantable to tissue culture. Transmission by filtrate, neg.	31	*44*

[26] The change from carcinoma to sarcoma occurred during transplantation to rats fed a choline-deficient diet.

II.—Lymphomas and Leukemias—Continued

II–E.—Lymphoid tumors and erythrogranuloblastosis of fowls [37]

Designation and histologic type Name in italics as modified by authors. Synonym in parentheses.	Origin			Host Species. Strain and subline when given (*60*).
	Spontaneous (*year if known*)	Induced (*year if known*)	Derivation	
Lymphoid tumor RPL12 (Olson lymphoid tumor)	1937 In nature		Liver and other sites	Chicken Not specific for breed
Lymphoid tumor RPL14	1944 In nature		Lung	Chicken Not specific for breed
Lymphoid tumor RPL15	1944 In nature		Spleen	Chicken Not specific for breed
Lymphoid tumor RPL16	1944 In nature		Liver	Chicken Not specific for breed
Lymphoid tumor RPL17	1944 In nature		Liver	Chicken Not specific for breed
Lymphoid tumor RPL18	1946 In nature		Ovary	Chicken Not specific for breed
Lymphoid tumor RPL19 Hemocytoblastic	1946 In nature		Ovary	Chicken Not specific for breed
Lymphoid tumor RPL20	1946 In nature		Spleen	Chicken Not specific for breed
Lymphoid tumor RPL21	1946 In nature		Liver	Chicken Not specific for breed

[37] Biester and Devries (*24*) and Foulds (*115*) discuss fowl tumors.

II.—Lymphomas and Leukemias—Continued

Growth characteristics				Special features	Contributor(s)	References
Takes (*percent*)	Latent period in days	Kills host in days	Infiltration and metastasis			
100 In young hosts	6	6–14	Widely disseminated.	Explantable to tissue culture and chick embryo. Transmission by filtrate, pos. Virus tumor. [28] [29]	12	*234, 235, 308*
100 In young hosts	6	10–21	Widely disseminated.	Virus tumor.	12	*47*
100 In young hosts	6	10–21	Widely disseminated.	Virus tumor.	12	*47*
100 In young hosts	6	6–14	Widely disseminated.	Transmission by filtrate, pos. Virus tumor. [28]	12	*47, 309*
90 In young hosts	7	7–28	Widely disseminated.	Virus tumor.	12	*47*
95 In young hosts	10	7–28	Widely disseminated.	Transmission by filtrate, pos. Virus tumor. [28]	12	*46, 309*
90 In young hosts	14	21–84	Widely disseminated.	Transmission by filtrate, pos. Virus tumor. [30]	12	*45, 46*
95 In young hosts	10	7–28	Widely disseminated.	Transmission by filtrate, pos. Virus tumor. [28]	12	*46*
100 In young hosts	10	7–28	Widely disseminated.	Transmission by filtrate, pos. Virus tumor. [28] [29]	12	*46*

[28] The figures for "Takes," "Latent period," and "Kills host" are for cellular transplants to pectoral muscle. When tumor or blood filtrates are injected intraperitoneally, there are 75 percent takes, and the latent period and killing time are greatly prolonged.

[29] The tumor is contaminated with a filtrable agent producing osteopetrosis in about 50 percent of the hosts

[30] Hemangiomatosis occurs in a subline.

II.—Lymphomas and Leukemias—Continued

Designation and histologic type Name in italics as modified by authors. Synonym in parentheses.	Origin			Host
	Spontaneous (*year if known*)	Induced (*year if known*)	Derivation	Species. Strain and subline when given (*60*).
Erythrogranuloblastosis RPL3 Erythroblastic↔myelocytic	1940 In nature		Blood	Chicken Not specific for breed
Erythrogranuloblastosis RPL4 Erythroblastic↔myelocytic	1939 In nature		Blood	Chicken Not specific for breed

III.—Tumors of Connective Tissue [32]

III–A.—Tumors and related lesions presumably of connective tissue origin and of specific cell type, such as fibrous tissue, [33] *fat, muscle, bone, and vascular tissue, and presumably originating in these tissues*

Fibrosarcoma S620		1948 20-Methylcholanthrene	Subcutaneous tissue	Mouse CBA/Sn and Jax; and hybrids
Fibrosarcoma S621		1948 20-Methylcholanthrene	Subcutaneous tissue	Mouse BALB/cSn and Jax; and hybrids
Fibrosarcoma S629		1948 20-Methylcholanthrene	Subcutaneous tissue	Mouse ST/Jax; and hybrids
Fibrosarcoma Sa89		1949 20-Methylcholanthrene	Subcutaneous tissue	Mouse C57BL
Fibrosarcoma S636		1948 20-Methyl cholanthrene	Subcutaneous tissue	Mouse C57BR/aSn; and hybrids
Fibrosarcoma HE8971		1950 β, β′-Dichlorodiethyl sulfide (Sulfur mustard)	Subcutaneous tissue	Mouse C3H/He

[32] Saxén and Stewart (*353*) discuss criteria for the diagnosis and classification of induced sarcomas.

[33] Sarcomas showing myxomatous change are listed with tumors of fibrous tissue.

II.—Lymphomas and Leukemias—Continued

Growth characteristics				Special features	Contributor(s)	References
Takes (*percent*)	Latent period in days	Kills host in days	Infiltration and metastasis			
75	5–10	12–44	Widely disseminated.	Explantable to chick embryo. Transmission by filtrate, pos. Virus tumor. [31]	12	*33, 307*
50	10–15	25–45	Widely disseminated.	Explantable to chick embryo. Transmission by filtrate, pos. Virus tumor.	12	*89, 307*

III.—Tumors of Connective Tissue

100	7	28–35			93	
100	3–4	21–28			93	*280, 284*
100	7	28–35			93	
100	5	14–16	Local invasion. Lung, rare.	No regression.	27, 96	*67*
100	3–4	21–28			93	
100	10	35–45			51	

[31] A so-called myxosarcoma variant is described.

III.—Tumors of Connective Tissue—Continued

Designation and histologic type Name in italics as modified by authors. Synonym in parentheses.	Origin			Host Species. Strain and subline when given (*60*).
	Spontaneous (*year if known*)	Induced (*year if known*)	Derivation	
Sarcoma Earle L (L Sarcoma) Fibrosarcoma		1942 20-Methylcholanthrene	Subcutaneous fibroblasts grown and treated *in vitro*	Mouse C3H/An
Fibrosarcoma Sa87		1948 20-Methylcholanthrene	Subcutaneous tissue	Mouse C3H
Sarcoma dbaG Fibrosarcoma	✓		Mammary gland, from transplanted carcinoma	Mouse DBA/Ha
Fibrosarcoma DS7		1948	Subcutaneous tissue	Mouse "ND"
Sarcoma DS8 Fibrosarcoma	1950		Subcutaneous tissue	Mouse "ND"
Fibrosarcoma BP 839		1936 3,4-Benzpyrene	Subcutaneous tissue	Rat A × C/9935 (Irish)
Fibrosarcoma ACMCA2		1946 20-Methylcholanthrene	Subcutaneous tissue	Rat A × C/9935 (Irish)
Fibrosarcoma R2572	✓		Mammary gland, from transplanted carcinosarcoma	Rat A × C/9935 (Irish)
Fibrosarcoma King A #231		1948 20-Methylcholanthrene	Subcutaneous tissue	Rat King A, inbred
Fibrosarcoma #7		1950 20-Methylcholanthrene	Subcutaneous tissue	Rat Lewis, inbred
Fibrosarcoma No. 8		1947 20-Methylcholanthrene	Subcutaneous tissue	Rat "Long-Evans/UCLA"

III.—Tumors of Connective Tissue—Continued

Growth characteristics				Special features	Contributor(s)	References
Takes (*percent*)	Latent period in days	Kills host in days	Infiltration and metastasis			
100	7	60	Local invasion. Lung, rare.	No regression. Explantable to tissue culture.	1, 23, 33, 110	*86, 88, 230, 261, 312*
90	14–21	63–91	Local invasion. Lung, 5%.	Necrosis. Regression, rare.	27, 96	
	5				45	
55–60		35–42	No metastasis observed.	No regression.	76, 90	
100		28–35	No metastasis observed.	No necrosis. No regression.	76, 90	
100	8	35–40	Local invasion. Metastasis, rare.	No regression.	31	*44*
100	7	30	Local invasion. No metastasis.	Necrosis, minimal. Large tumor growth.	4, 66	*210*
100	8	28–35	Local invasion. Lymph nodes. Lung.	No regression. Explantable to tissue culture.	31, 45	*79*
100		15–20	Local invasion. No metastasis.		61	*171*
100		28–35	Local invasion. No metastasis.		61	
90		28	Local invasion, rare. No metastasis.		29	*74, 220*

III.—Tumors of Connective Tissue—Continued

Designation and histologic type Name in italics as modified by authors. Synonym in parentheses.	Origin			Host Species. Strain and subline when given (*60*).
	Spontaneous (*year if known*)	Induced (*year if known*)	Derivation	
Fibrosarcoma JS1		1947 20-Methylcholanthrene.	Subcutaneous tissue	Rat "Curtis-Dunning/Donner"
Fibroma Emge	1929		Mammary gland, from transplanted adenofibroma	Rat Wistar, Stanford
Fibrosarcoma Noble	1949		Mammary gland, from transplanted fibroadenoma	"Rat"
Fibrosarcoma Friedewald		1950 20-Methylcholanthrene	Subcutaneous tissue	Hamster Syrian
Sarcoma HS5 (No. 5) Myxosarcoma	1950		"Connective tissue"	Hamster Syrian, inbred
Infectious fibroma Shope	1931 In nature		Skin	Rabbit Cottontail
Infectious myxoma	1896 In nature		"Connective tissue"	Rabbit Domestic (*Oryctolagus*)
Tumor C Fibrosarcoma	1942 In nature		Pectoral region	Chicken Rhode Island red, Plymouth rock

III.—Tumors of Connective Tissue—Continued

Growth characteristics				Special features	Contributor(s)	References
Takes (*percent*)	Latent period in days	Kills host in days	Infiltration and metastasis			
100	10	42	No metastasis observed.		52	*259*
50–100	28–42	(Does not kill)	No invasion.	Large tumor growth. No regression. Hyalinization. Benign and malignant forms.[34]	36	*97*
80–90		28–42		No necrosis. Benign and malignant forms.[35]	74	*70*
100	7–14	14–28	Local invasion. No metastasis observed.		42	*231*
100	35	(Does not kill)	No metastasis observed.	Necrosis, minimal. Large tumor growth.	25	
100	5	(Does not kill)	No invasion.	Regression, in all cases. Explantable to chick embryo. Transmission by filtrate, pos. Virus tumor.[36]	89	*1, 82, 169, 270*
100	3–5	8–10	Invasion of skin and subcutaneous tissues.	Explantable. Transmission by filtrate, pos. Virus tumor.[36]	89	*107, 169, 181, 251, 271*
82 In young hosts	"a few"	"many months"	Metastasis, rare.	No regression. Transmission by filtrate, pos. Virus tumor.	32	*84*

[34] For related tumors, see *Adenofibroma Emge*, page 1356, and spindle-cell *Sarcoma Emge*, page 1352.

[35] The growth rate is not affected by hormones, or by the protein content of the diet. Contrast with *Fibroadenoma tumour #6*, page 1356.

[36] The myxoma is a virus inflammatory lesion which in resistant hosts may approach the fibroma type of lesion. Otherwise it spreads widely in the subcutaneous tissues and kills most of the animals. The fibroma may be looked upon as a lesion intermediate between the myxoma and true neoplasia.

III.—Tumors of Connective Tissue—Continued

Designation and histologic type Name in italics as modified by authors. Synonym in parentheses.	Origin			Host Species. Strain and subline when given (60).
	Spontaneous (*year if known*)	Induced (*year if known*)	Derivation	
Chicken tumor I (Rous sarcoma) Fibrosarcoma and myxoma	1910 In nature		Subcutaneous tissue	Chicken Grows in most strains
Myxosarcoma 14(d)7 Fibrosarcoma		1945 Adaptation of virus	By subcutaneous inoculation of cells of *Chicken Tumor I*	Pekin duck
Fibrosarcoma Sa27		1950 20-Methylcholanthrene	"Forelimb"	Newt (*Triturus viridescens*)
Liposarcoma D4888		1945 20-Methylcholanthrene	Subcutaneous tissue	Guinea pig Strain 2 (Family 2)
Rhabdomyosarcoma H6668	1951		Muscle	Mouse BALB/cHu
Rhabdomyosarcoma MCIA		1945 20-Methylcholanthrene	Muscle	Mouse C3H/St and Thompson
Sarcoma MCIM		1946 20-Methylcholanthrene	Muscle, from transplanted rhabdomyosarcoma	Mouse C3H/St
Rhabdomyosarcoma S653		1949 20-Methylcholanthrene	Subcutaneous tissue	Mouse C57BL/10; and hybrids

III.—Tumors of Connective Tissue—Continued

Growth characteristics				Special features	Contributor(s)	References
Takes (*percent*)	Latent period in days	Kills host in days	Infiltration and metastasis			
90–100 In young hosts	3–5	10–21	Local invasion. Heart, liver, lung.	Necrosis. Hemorrhage. Regression, rare. Explantable to tissue culture and chick embryo. Transmission by filtrate, pos. Virus tumor.	10	*39, 41, 113, 245, 246, 298*
100 In young hosts	"a few"	5–20	Local invasion. Metastasis.	Hemorrhage. No regression. Transmission by filtrate, pos. Virus tumor. [37]	32	*32, 83*
60	30–90	120–400	Local invasion.	Regression, rare. Explantable to tissue culture. Transmission by filtrate, neg. [38]	8	*34*
100	10	35	Lung.	Necrosis.	51	*154*
100	7	50	Local invasion.	Tumor cells show cross-striations.	40	
100	14	30–60	Local invasion. Lung.	Necrosis. No regression. Cross-striation rare in tumor cells. Ascites tumor.	46, 50, 57	*151, 176, 217, 218*
100		15–20		No regression. Undifferentiated. Ascites tumor.	50, 57	*176*
100	7–9	28–35		Undifferentiated.	93, 108	

[37] The tumor was produced by adaptation of a chicken tumor virus to the duck. Successful transmission of the tumor to pigeons has been described.

[38] A myxomatous change occurs in some growths. Cells of the *Triturus* newt are larger than those of mammals and possess a smaller number of chromosomes.

III.—Tumors of Connective Tissue—Continued

Designation and histologic type Name in italics as modified by authors. Synonym in parentheses.	Origin			Host Species. Strain and subline when given (*60*).
	Spontaneous (*year if known*)	Induced (*year if known*)	Derivation	
Sarcoma R92	1922		Sternum, from transplanted chondrorhabdomyosarcoma	Rat Zimmerman/61
Rhabdomyosarcoma HS4		1949 9,10-Dimethyl-1,2-benzanthracene	Subcutaneous tissue	Hamster Syrian, inbred
Bone tumor #4 NCI	1952		Femur	Mouse $C3H_1/He$
Osteogenic sarcoma Wagner (WA sarcoma)	1947		Supraclavicular region	Mouse AKR/M and Jax; and hybrids
Osteogenic sarcoma Ridgway	1948		Inguinal region	Mouse AKR/M
Osteogenic sarcoma T491	1951 X radiation		Shoulder region	Mouse C57BL/Yale
Sarcoma #4 Argonne	1947 Spontaneous?		Bone, from transplanted osteogenic sarcoma	Mouse CF1 (Carworth)

III.—Tumors of Connective Tissue—Continued

Growth characteristics				Special features	Contributor(s)	References
Takes (*percent*)	Latent period in days	Kills host in days	Infiltration and metastasis			
100	8	30–35	Local invasion. Regional nodes. Lung.	No regression. Explantable, to tissue culture. Transmission by filtrate, neg. Undifferentiated.	31	*42*
100		60	Local invasion. Lymph nodes. Lung.	Necrosis.	25	
100	8–10	30–40	Local invasion. Lymph nodes. Lung.	No regression. Bone formation in intramuscular transplants. High acid and alkaline phosphatase.	39	*17*
99–100	5–10	35–70	Local invasion. Metastasis, rare.	Necrosis, minimal. 6% regression. Explantable. Transmission by filtrate, neg. No bone formation. High alkaline phosphatase. Undifferentiated.	93, 100	*176, 300*
99	4	21–42	Metastasis, rare.	Necrosis, minimal. 3% regression. Explantable. Transmission by filtrate, neg. No bone formation. High alkaline phosphatase. Undifferentiated.	100	*221, 300*
100					53	
30	10–14	30–60		Undifferentiated.	9	

III.—Tumors of Connective Tissue—Continued

Designation and histologic type Name in italics as modified by authors. Synonym in parentheses.	Origin			Host Species. Strain and subline when given (60).
	Spontaneous (*year if known*)	Induced (*year if known*)	Derivation	
Osteogenic sarcoma #344		1950 20-Methylcholanthrene	Stomach	Rat M520 (Marshall)
Hemangioendothelioma H6221	1951		Epididymis	Mouse BALB/cHu
III–B.—Tumors presumably of connective tissue origin and of cell type not specified otherwise than as "mixed cell," "pleomorphic," "round cell," "fusiform cell," "spindle cell," "anaplastic," "undifferentiated," or "sarcoma"				
Sarcoma C3H (No. 2) Round cell	1949		Mammary gland, from transplanted carcinoma	Mouse C3H/Dm
Sarcoma B12 (L.E.O.) Spindle cell		1950 3,4-Benzpyrene, from barnacles	Subcutaneous tissue	Mouse C3H/An
Sarcoma I (SI) Spindle cell		1947 1,2,5,6-Dibenzanthracene	Subcutaneous tissue	Mouse A/Jax; and hybrids
Sarcoma A19E Pleomorphic cell	1950		Mammary gland, from transplanted carcinoma	Mouse A/Dm
Sarcoma T241 Lewis Pleomorphic cell		1938 1,2,5,6-Dibenzanthracene	Subcutaneous tissue, from transplanted fibrosarcoma	Mouse C57BL
Sarcoma Ma387 Fusiform cell	1942		Lung, from transplanted adenoma	Mouse AKR/M

III.—Tumors of Connective Tissue—Continued

Growth characteristics				Special features	Contributor(s)	References
Takes (*percent*)	Latent period in days	Kills host in days	Infiltration and metastasis			
100	7–10	60–90		No regression. Little bone formation. High acid and alkaline phosphatase. Undifferentiated.	97	
80	8–10	28–42		Hemorrhage.	40	
100	7			Hemorrhage. Explantable to tissue culture. Transmission by filtrate, neg.	28	*72, 236, 237*
100		42	Local invasion.		88	*269*
100	2–7	21	No metastasis observed.	Necrosis.	40, 52	
100	7			Hemorrhage. Explantable to tissue culture. Transmission by filtrate, neg.	28	
100	4–7	21–35	Local invasion. Lung, rare.	Necrosis, extensive. No regression. Explantable to tissue culture. Transmission by filtrate, neg. Undifferentiated.	20,100, 111	*176, 206, 221, 300*
100	4	21–42	Metastasis, rare.	Necrosis, extensive. No regression. Explantable. Transmission by filtrate, neg.	100	*36, 300*

III.—Tumors of Connective Tissue—Continued

Designation and histologic type Name in italics as modified by authors. Synonym in parentheses.	Origin			Host Species. Strain and subline when given (*60*).
	Spontaneous (*year if known*)	Induced (*year if known*)	Derivation	
Sarcoma RIII (No. 4) Spindle cell	1949		Mammary gland, from transplanted carcinoma	Mouse RIII/Dm
Sarcoma S637 Anaplastic cell		1948 20-Methylcholanthrene	Subcutaneous tissue	Mouse P/Sn and Jax; and hybrids
Sarcoma DS2 Spindle cell		1949	Subcutaneous tissue	Mouse "ND"
Sarcoma DS3 Spindle cell		1949	Subcutaneous tissue	Mouse "ND"
Sarcoma DS4 Pleomorphic cell		1949	Subcutaneous tissue	Mouse "ND"
Sarcoma DS5 Spindle cell		1949	Subcutaneous tissue	Mouse "ND"
Sarcoma 37 Pleomorphic cell	1906		Mammary gland, from transplanted carcinoma	Mouse Not restricted to a particular strain
Sarcoma 180 Crocker Pleomorphic cell	1914		Region of axilla (male host), from transplanted carcinoma	Mouse Not restricted to a particular strain
Sarcoma A274 Undifferentiated cell		1938 1,2,5,6-Dibenzanthracene		Mouse A; and hybrids

III.—Tumors of Connective Tissue—Continued

Growth characteristics				Special features	Contributor(s)	References
Takes (*percent*)	Latent period in days	Kills host in days	Infiltration and metastasis			
100	7			Hemorrhage. Explantable to tissue culture. Transmission by filtrate, neg.	28	*236*
100	5–7	28–35		Anaplastic.	93	*284*
65		84–112	No metastasis observed.	No necrosis. No regression.	76, 90	
100		28–35	No metastasis observed.	Necrosis. No regression.	76, 90	
100		35–56	No metastasis observed.	No regression. Undifferentiated.	76, 90	
100		28–35	No metastasis observed.		76, 90	
100	Solid form: 3–8 Ascites form: 3–4	Solid form: 18–49 Ascites form: 8–14	Local invasion. Lymph nodes. Lung, occasional.	Necrosis, extensive. Regression, some strains. Transmission by filtrate, neg. Undifferentiated. Ascites tumor. [39]	15, 35, 40, 41, 48, 50, 52, 85	*5, 20, 134, 135, 147, 151, 176, 311, 312*
90–100	3–5	14–28	Local invasion. Lung, occasional.	Necrosis, extensive. 6% regression, after ulceration. Explantable. Transmission by filtrate, neg. Anaplastic.	27, 40, 82, 96, 100, 101, 104, 107	*23, 52, 115, 141, 157, 176, 222, 252, 294, 300, 312, 318*
100	5–7	21–28	No metastasis observed.	Necrosis. Undifferentiated.	52	*239*

[39] From the evidence from the literature the original tumor was a mammary carcinoma, which during serial transplantations underwent successive changes to mixed-cell malignant tumor, to fibrosarcoma, to pleomorphic cell sarcoma.

III.—Tumors of Connective Tissue—Continued

Designation and histologic type Name in italics as modified by authors. Synonym in parentheses.	Origin			Host Species. Strain and subline when given (60).
	Spontaneous (*year if known*)	Induced (*year if known*)	Derivation	
Sarcoma MCI Undifferentiated cell		1948 20-Methylcholanthrene	Subcutaneous tissue	Mouse Not restricted to a particular strain
Sarcoma IRS 1548 Pleomorphic cell		1926 *Cysticercus fasciolaris*	Subcutaneous tissue	Rat Fischer/344
Sarcoma IRS 4337 Spindle cell		1932 *Cysticercus fasciolaris*	Cyst of liver	Rat Copenhagen/2331
Sarcoma 1643 Fusiform cell		1946 2-Diacetylaminofluorene	Region of groin, from transplanted carcinoma	Rat Buffalo
Sarcoma E2730		1947	Subcutaneous tissue	Rat M520 (Marshall)
Sarcoma #3 Lewis		1946 20-Methylcholanthrene	Subcutaneous tissue	Rat Lewis, inbred
Sarcoma M4	1943		From transplanted lymphosarcoma	Rat Slonaker
Sarcoma Emge Spindle cell	1932 and later		Mammary gland, from transplanted adenofibroma to fibroma	Rat Wistar, Stanford
Sarcoma #319 Walker	1929		Perineal region soft tissue	Rat "Albino"

III.—Tumors of Connective Tissue—Continued

Growth characteristics				Special features	Contributor(s)	References
Takes (*percent*)	Latent period in days	Kills host in days	Infiltration and metastasis			
100	5–6	14–21	No metastasis observed.	Necrosis. 10% regression. Undifferentiated.	70	
99	14	42–56	Local invasion. No metastasis observed.	Necrosis. Regression, after ulceration. Explantable to tissue culture. Transmission by filtrate, neg.	31	*43*
100	7	30–40	Local invasion. Lymph nodes. Lung.	No regression. Explantable to tissue culture. Transmission by filtrate, neg.	31	*78, 209, 213*
100		70	Local invasion.		71	*144, 210, 226, 244*
					34	*210*
100		21–28	Local invasion. No metastasis observed.		61	
100	10–14	30	Necrosis.		52	*260*
100	3–6	30–40	No metastasis observed.	Necrosis. No regression. Benign and malignant forms. [40]	36	*69, 97, 98*
60	7	60	Local invasion.	Necrosis. Explantable to tissue culture. [41]	75	*129, 193, 241*

[40] For related tumors, see *Adenofibroma Emge*, page 1356, and *Fibroma Emge*, page 1342.

[41] The multipolar cell, seen in tissue cultures of the tumor, resembles a neuron;

III.—Tumors of Connective Tissue—Continued

Designation and histologic type Name in italics as modified by authors. Synonym in parentheses.	Origin			Host Species. Strain and subline when given (*80*).
	Spontaneous (*year if known*)	Induced (*year if known*)	Derivation	
Sarcoma Jensen Spindle cell	1907 In host inoculated with acid-fast bacilli		Peritoneal and metastatic tumor nodules	Rat Sprague-Dawley-Holtzman Wistar Sherman
Sarcoma HS6 Pleomorphic cell		1944 9,10-Dimethyl-1, 2-benzanthracene	Sub-pannicular region	Hamster Syrian, inbred

IV.—Tumors of Melanin-Forming Tissue [43]

Melanoma Harding-Passey	1925		Ear	Mouse Not restricted to a particular strain
Melanoma S91 Cloudman (dba melanoma)	1937		Base of tail	Mouse DBA/1Jax
Amelanotic melanoma S91A (C91AA)		1942	Base of tail, from transplanted *Melanoma S91*	Mouse BALB/c
Melanotic tumor Brunst (Chromatophoroma malignum)	1932 In nature		Pigment cells of corium	Axolotl (*Siredon mexicanum*)

[43] Miner and Gordon (*#19*) discuss melanotic tumors of animals.

III.—Tumors of Connective Tissue—Continued

Growth characteristics				Special features	Contributor(s)	References
Takes (*percent*)	Latent period in days	Kills host in days	Infiltration and metastasis			
80–98	5–6	17–56	Local invasion. Metastasis, rare.	Necrosis, extensive. 18% regression. Explantable. Transmission by filtrate, neg.	33, 80, 100	*49, 87, 162, 297, 300*
100		19–45	Local invasion. Lymph nodes. Lung, kidney.	No regression. [42]	25	*63, 64, 65, 166, 167*

IV.—Tumors of Melanin-Forming Tissue [43]

90–100	7–28	120–150	No invasion.	3% regression. Explantable to tissue culture. Transmission by filtrate, neg. [44]	1, 60, 63, 100	*20, 149, 176, 295, 296, 300, 319*
40–100	7–28	42–90	Local invasion. Lymph nodes. Lung, frequent.	Necrosis, minimal. Regression, rare. Explantable to tissue culture. Transmission by filtrate, neg. Ascites tumor. [45]	1, 27, 40, 48, 59, 60, 63, 83, 96	*3, 57, 144, 176, 201, 283, 286, 319*
50–70	28–42	120	Local invasion. Lymph nodes. Lung, rare.	Necrosis. Transmission by filtrate, neg. [46]	1, 59, 63	*3, 201, 319*
100		400–600	Invasion throughout corium.		87	*267*

[43] A similar tumor, *Sarcoma lung line 6*, was derived through transplantation of metastases. Periportal infiltration of lymphocytes and plasmacytes in liver, and extramedullary hematopoiesis were observed in hamsters bearing the sarcomas.

[44] The tumor is composed of two distinctive cell types, one a dendritic melanoblast, the other a macrophage or melanophore that constitutes 50 to 80 percent of the tumor.

[45] The cells are pleomorphic and sarcoma-like with considerable variations in pigment content. The tumor may become amelanotic following transplantation, as stated by Schrek.

[46] The tumor, produced by adaptation of a melanotic tumor to an albino host, is grossly amelanotic. Spindle cells predominate, and occasionally pigment granules can be seen within the cells.

V.—Tumors of Nervous Tissue

Designation and histologic type Name in italics as modified by authors Synonym in parentheses.	Origin			Host
	Spontaneous (*year if known*)	Induced (*year if known*)	Derivation	Species. Strain and subline when given (*60*).
Astrocytoma C3H (*18*)		1948 20-Methylcholanthrene	Brain	Mouse C3H/St
Ependymoma A(*22*)		1947 20-Methylcholanthrene	Brain	Mouse A/St
Glioblastoma 8110 Moore		1949 20-Methylcholanthrene	Brain	Mouse A
Neuroblastoma C1300	1940		Region of spinal cord	Mouse A/Jax; and hybrids "ABC"

VI.—Tumors Composed of Mixed Tissues

VI–A.—Tumors composed of mixed epithelial and connective tissues (fibroadenoma and carcinosarcoma)

Fibroadenoma R2737	1946		Mammary gland	Rat August/28807
Adenofibroma Emge	1928		Mammary gland	Rat Wistar, Stanford
Fibroadenoma tumour #6	1949		Mammary gland	"Rat"
Carcinosarcoma 256 Walker (Walker rat tumor)	1928		Probably mammary gland, from transplanted carcinoma	Rat Not restricted to a particular strain

V.—Tumors of Nervous Tissue

Growth characteristics				Special features	Contributor(s)	References
Takes (*percent*)	Latent period in days	Kills host in days	Infiltration and metastasis			
100			Local invasion. No metastasis.	Tumor is cystic. Explantable to chick embryo.	112	*324, 325*
100			Local invasion. No metastasis.	Explantable to chick embryo.	112	*324, 325*
85	12–14	75–90	No metastasis.	Explantable. Transmission by filtrate, neg.	107	
100	4–10	14–70	Local invasion. Lymph nodes.	Classification in doubt: "possibly a neuroblastoma."	35, 40, 66, 94	*91, 92, 138, 176, 221, 285*

VI.—Tumors Composed of Mixed Tissues

100 In female hosts.	21–28	300 or more	No invasion.	No regression. Hormone excites secretion. [47]	31	*198*
50–100	14–28	(Does not kill)	No invasion.	No regression. Hyalinization. Benign and malignant forms. [48]	36	*69, 96*
80–90	28–42		No invasion.	Benign and malignant forms. [49]	74	*70*
80–100	2–10	14–70	Local invasion. Regional nodes. Lung, rare.	Necrosis. Hemorrhage. Regression, rare. Explantable to tissue culture and chick embryo. Transmission by filtrate, neg.	3, 20, 33, 65, 67, 69, 80, 83, 94, 98, 100, 101	*49, 85, 142, 153, 215, 216, 254, 258, 268, 300, 305*

[47] The tumor is encapsulated, attaining large dimensions before the animal dies, and undergoes secondary infection, hemorrhage, or ulceration. It is possible to remove the tumor without recurrence.

[48] For related tumors, see *Fibroma Emge*, page 1342, and *Sarcoma Emge*, page 1352.

[49] Ten percent of the tumors develop sarcomatous changes (see *Fibrosarcoma Noble*, page 1342). The growth rate is affected by hormone administration and, probably, by dietary protein. Transplantation is by tumor slices.

VI.—Tumors Composed of Mixed Tissues—Continued

VI–B.—Teratomas

Designation and histologic type Name in italics as modified by authors. Synonym in parentheses.	Origin			Host
	Spontaneous (*year if known*)	Induced (*year if known*)	Derivation	Species. Strain and subline when given (*99*).
Embryoma Brues-Jackson	1940		Ovary	Mouse C3H/An
Teratoma E6496	1951		Ovary	Mouse C3H.C57BL

VI.—Tumors Composed of Mixed Tissues—Continued

Growth characteristics				Special features	Contributor(s)	References
Takes (*percent*)	Latent period in days	Kills host in days	Infiltration and metastasis			
75–90	7–10	60–180	No metastasis observed.	No necrosis. Explantable to tissue culture. Transmission by filtrate, neg. Hormonally inactive.	2, 9	*159*
100	7–9	42–49	Local invasion. No metastasis observed.	Explantable to tissue culture.	40	*104*

Contributors

The numbers in the column of the table entitled "Contributors" correspond to the numbered list below.

Contributors in most cases have sent in detailed data on transplantable or transmissible tumors maintained in their laboratories, or in a few other cases have written that they maintained certain tumors but have sent no data. Although a few investigators have written that they no longer maintained the tumor reported, they were so familiar with it in the past that their data have been used. The contributor's laboratory is not necessarily the laboratory of origin of a tumor.

A list of the contributors follows:

1. Glenn H. Algire, National Cancer Institute, Bethesda 14, Md.
2. Joseph C. Aub, Massachusetts General Hospital, Boston 14, Mass.
3. Burton L. Baker, University of Michigan Medical School, Ann Arbor, Mich.
4. Morris K. Barrett, National Cancer Institute, Bethesda 14, Md.
5. Jørgen Bichel, Radiumstationen for Jutland, Aarhus, Denmark.
6. Georgiana M. Bonser, University of Leeds, School of Medicine, Leeds 2, England.
7. Zybněk Brada, Cancer Hospital, Žlutý kopec 7, Brno, Czechoslovakia.
8. Charles Breedis, University of Pennsylvania, The School of Medicine, Philadelphia 4, Pa.
9. Austin M. Brues, Argonne National Laboratory, Chicago 80, Ill.
10. W. Ray Bryan, National Cancer Institute, Bethesda 14, Md.
11. Dean Burk, National Cancer Institute, Bethesda 14, Md.
12. Ben R. Burmester, Regional Poultry Research Laboratory, Bureau of Animal Industry, Box 270, East Lansing, Mich.
13. Robert H. Butler, University of Nebraska College of Medicine, Omaha 5, Nebr.
14. Perihan Cambel, Cancer Research Laboratory, University of Florida, Gainesville, Fla. (*Present address*: Birincicadde 212, Istanbul-Arnavutkoey, Turkey.)
15. Antonio Cantero, Montreal Cancer Institute, Montreal, Quebec, Canada.
16. Charles M. Carpenter, University of California School of Medicine, Los Angeles 24, Calif.
17. Albert E. Casey, Baptist Hospital, Birmingham 9, Ala.
18. Joseph K. Cline, Medical College of Alabama, Birmingham 5, Ala.
19. Irvin J. Cohen, Veterans Administration Hospital, Bronx 63, N. Y.
20. Dale R. Coman, University of Pennsylvania, The School of Medicine, Philadelphia 4, Pa.
21. Charles C. Congdon, National Cancer Institute, Bethesda 14, Md.
22. Donovan H. Copeland, Alabama Polytechnic Institute, Auburn, Ala.
23. Ivor Cornman, George Washington University Cancer Clinic, Washington 5, D. C.
24. Edmund V. Cowdry, Washington University School of Medicine, St. Louis 10, Mo.
25. Edward D. Crabb, Denison Laboratory, University of Colorado, Boulder, Colo.
26. Albert J. Dalton, National Cancer Institute, Bethesda 14, Md.
27. Israel Davidsohn, Mount Sinai Medical Research Foundation, Chicago 8, Ill.
28. Leon Dmochowski, University of Leeds, School of Medicine, Leeds 2, England.
29. Max S. Dunn, Department of Chemistry, University of California, Los Angeles 24, Calif.
30. Thelma B. Dunn, National Cancer Institute, Bethesda 14, Md.
31. Wilhelmina F. Dunning, The University of Miami, Coral Gables (University Branch) 46, Fla.
32. Francisco Duran-Reynals, Department of Microbiology, Yale University, New Haven 11, Conn.
33. Wilton R. Earle, National Cancer Institute, Bethesda 14, Md.
34. Harold E. Eggers, University of Nebraska College of Medicine, Omaha 5, Nebr.
35. Ernst J. Eichwald, Salt Lake General Hospital, Salt Lake City 15, Utah.
36. Ludwig A. Emge, Stanford University School of Medicine, San Francisco 15, Calif.
37. Ralph L. Engle, Jr., The New York Hospital, New York 21, N. Y.

38. Allen Eschenbrenner, National Cancer Institute, Bethesda 14, Md.
39. Willard H. Eyestone, National Cancer Institute, Bethesda 14, Md.
40. Elizabeth Fekete, Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.
41. Marie D. Felix, National Cancer Institute, Bethesda 14, Md.
42. William F. Friedewald, Emory University School of Medicine, Atlanta 3, Ga.
43. Jacob Furth, Oak Ridge National Laboratory, Oak Ridge, Tenn.
44. William U. Gardner, Yale University School of Medicine, New Haven 11, Conn.
45. Anna Goldfeder, New York University College of Medicine, New York 16, N. Y.
46. Elizabeth U. Green, The Institute for Cancer Research, Philadelphia 11, Pa.
47. Robert E. Greenfield, Jr., National Cancer Institute, Bethesda 14, Md.
48. Paul F. Hahn, Meharry Medical College, Nashville 8, Tenn.
49. William M. Hale, Brookhaven National Laboratory, Upton, Long Island, N. Y.
50. Theodore S. Hauschka, The Institute for Cancer Research, Philadelphia 11, Pa.
51. Walter E. Heston, National Cancer Institute, Bethesda 14, Md.
52. Hardin B. Jones, Donner Laboratory, University of California, Berkeley 4, Calif.
53. Henry S. Kaplan, Stanford University School of Medicine, San Francisco 15, Calif.
54. John G. Kidd, Cornell University Medical College, New York 21, N. Y.
55. Riojun Kinosita, University of California School of Medicine, Los Angeles 24, Calif.
56. Arthur Kirschbaum, University of Illinois College of Medicine, Chicago 12, Ill.
57. George Klein, Karolinska Institutet, Stockholm 60, Sweden.
58. Lloyd W. Law, National Cancer Institute, Bethesda 14, Md.
59. Edwin A. Lawrence, Indiana University Medical Center, Indianapolis 7, Ind.
60. A. Bunsen Lerner, University Hospital, Ann Arbor, Mich.
61. Margaret R. Lewis, The Wistar Institute of Anatomy and Biology, Philadelphia 4, Pa.
62. Egon Lorenz, National Cancer Institute, Bethesda 14, Md.
63. Prosper Loustalot, Ciba Company, Ltd., Basel, Switzerland.
64. Balduin Lucké, University of Pennsylvania, The School of Medicine, Philadelphia 4, Pa.
65. Clarence C. Lushbaugh, Los Alamos Scientific Laboratories, Los Alamos, N. Mex.
66. Ian Macdonald, University of Southern California School of Medicine, Los Angeles 33, Calif.
67. Harold D. McEwen, Queens University, Kingston, Ontario, Canada.
68. Mary E. Maver, National Cancer Institute, Bethesda 14, Md.
69. G. Burroughs Mider, National Cancer Institute, Bethesda 14, Md.
70. Alice E. Moore, The Sloan-Kettering Institute for Cancer Research, New York 21, N. Y.
71. Harold P. Morris, National Cancer Institute, Bethesda 14, Md.
72. Edwin D. Murphy, National Cancer Institute, Bethesda 14, Md.
73. Berne L. Newton, Baylor University College of Medicine, Houston 5, Tex.
74. Robert L. Noble, The University of Western Ontario, London, Ontario, Canada.
75. Charles M. Pomerat, The University of Texas, Medical Branch, Galveston, Tex.
76. William Prychodko, Detroit Institute of Cancer Research, Detroit 1, Mich.
77. Francis E. Ray, Cancer Research Laboratory, University of Florida, Gainesville, Fla.
78. Vernon T. Riley, National Cancer Institute, Bethesda 14, Md.
79. Peyton Rous, The Rockefeller Institute for Medical Research, New York 21, N. Y.
80. Harold P. Rusch, McArdle Memorial Laboratory, The University of Wisconsin, Madison 6, Wis.
81. Abe M. Schechtman, Department of Zoology, University of California, Los Angeles 24, Calif.
82. Emmanuel B. Schoenbach, The Johns Hopkins University School of Medicine, Baltimore 5, Md. (*Deceased*)
83. Robert Schrek, Veterans Administration Hospital, Hines, Ill.

84. Harry Shay, Fels Research Institute, Temple University School of Medicine, Philadelphia 40, Pa.
85. Murray J. Shear, National Cancer Institute, Bethesda 14, Md.
86. Emma Shelton, National Cancer Institute, Bethesda 14, Md.
87. E. A. Sheremetieva-Brunst, University of Maryland School of Medicine, Baltimore 1, Md.
88. Michael B. Shimkin, Laboratory of Experimental Oncology of the National Cancer Institute, Laguna Honda Home, San Francisco 16, Calif.
89. Richard E. Shope, Merck and Company, Rahway, N. J.
90. William L. Simpson, Detroit Institute of Cancer Research, Detroit 1, Mich.
91. Howard E. Skipper, Southern Research Institute, Birmingham 5, Ala.
92. Albert C. Snell, Jr., Strong Memorial Hospital, Rochester 20, N. Y.
93. George D. Snell, Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.
94. Sheldon C. Sommers, New England Deaconess Hospital, Boston 15, Mass.
95. Joseph Stasney, Jefferson Medical College of Philadelphia, Philadelphia 7, Pa.
96. Kurt Stern, Mount Sinai Medical Research Foundation, Chicago 8, Ill.
97. Harold L. Stewart, National Cancer Institute, Bethesda 14, Md.
98. John B. Storer, Los Alamos Scientific Laboratories, Los Alamos, N. Mex.
99. Ernest Sturm, The Rockefeller Institute for Medical Research, New York 21, N. Y.
100. Kanematsu Sugiura, The Sloan-Kettering Institute for Cancer Research, New York 21, N. Y.
101. James M. Surver, Jefferson Medical College of Philadelphia, Philadelphia 7, Pa.
102. Alexander Symeonidis, National Cancer Institute, Bethesda 14, Md.
103. Helene W. Toolan, The Sloan-Kettering Institute for Cancer Research, New York 21, N. Y.
104. Gray H. Twombly, Columbia University College of Physicians and Surgeons, New York 32, N. Y.
105. Donald R. Webster, McGill University Faculty of Medicine, Montreal, Quebec, Canada.
106. Albert H. Wheeler, University Hospital, Ann Arbor, Mich.
107. James H. Williams, Lederle Laboratories Division, American Cynamid Company, Pearl River, N. Y.
108. George W. Woolley, The Sloan-Kettering Institute for Cancer Research, New York 21, N. Y.
109. Tomizo Yoshida, The University of Tokyo Medical School, Hongo, Tokyo, Japan.
110. Julius S. Youngner, The University of Pittsburgh School of Medicine, Pittsburgh 13, Pa.
111. Irving Zeidman, University of Pennsylvania, The School of Medicine, Philadelphia 4, Pa.
112. Harry Zimmerman, Montefiore Hospital, New York 67, N. Y.

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The italic numbers in the column of the table entitled "References" correspond to the numbered list below.

In some cases reference is to a similar type of tumor, and not necessarily to the tumor named.

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The Society of General Physiologists

SCIENTIFIC PROCEEDINGS AND MINUTES

OF THE

SEVENTH ANNUAL MEETING

Ithaca, New York

September 9 and 10, 1952

1379

Foreword

What Is General Physiology?

It is desirable in any definition of general physiology to state first what general physiology is not. General physiology is not physiology in general. It is not concerned primarily with processes at the organ or system level. It is not elementary or introductory physiology. It is not concerned with the physiology of specialized groups of individual organisms, such as man, bacteria, plants, protozoa or domestic animals. There are other organized disciplines in the field of physiology to cover all the above categories.

General physiology may be defined as that branch of physiology which deals with all fundamental processes which occur in most but not necessarily in every living thing. These processes involve the physical and chemical reactions at the cellular, intercellular and subcellular level. The fundamental problem is to explain, in so far as possible, the nature and mechanism of living matter. Because general physiology is concerned with many functions which occur at the molecular level, it borders closely on biophysics and biochemistry, and may not be separated from them. Similarly it is not possible to separate general physiology from cytomorphology, because an explanation of function must be based upon structure as well as upon physics and chemistry. Briefly, general physiology is an integration of these three disciplines, directed toward the most fundamental problem in biology, namely, an understanding of protoplasmic structure, function and behavior.

The above definition of General Physiology contained in a report by Dr. Theodore L. Jahn, of the University of California, Los Angeles, seemed to the Council of the Society so apt and timely that it is printed herewith as an addition to the Proceedings of the Annual Meeting.

Minutes of the Society of General Physiologists

Annual Meeting—1952

Cornell University, Ithaca, New York

September 10, 1952

At the conclusion of the two-day meeting, consisting of a symposium and a presentation of individual papers, the annual business meeting was held with President L. R. Blinks presiding. The annual symposium was ably organized by Dr. Arthur C. Giese on the subject: "Contributions of Comparative Physiology to General Physiology." Invited papers were contributed by Drs. Robert E. Hungate, Lawrence R. Blinks, David R. Goddard, C. Ladd Prosser, Frank A. Brown, and Theodore H. Bullock.

AMENDMENTS TO THE CONSTITUTION: The two amendments to the Constitution submitted by ballot to the membership were affirmed by a vote of 81 to 27 and by 102 to 3 respectively. The first amendment provides that the Vice-President shall automatically be the President-elect for the ensuing year. The second makes the Representative to the AIBS Governing Board an ex-officio but nonvoting member of our Council and provides that he shall be elected by the Society every four years. By a vote of 83 to 22, it was decided that both amendments should apply to present officers.

ELECTION OF OFFICERS: It was announced that, following elections by the entire membership, the following list of officers will hold office as indicated below:

President:	*Dr. H. Burr Steinbach* (1-year term), Department of Zoology, University of Minnesota, Minneapolis, Minnesota.
Vice-President:	*Dr. Arthur K. Parpart* (1-year term), Department of Biology, Princeton University, Princeton, New Jersey. Votes for Parpart—67; Chance—38.
Secretary-Treasurer:	*Dr. William R. Duryee* (1 additional year), National Cancer Institute; *Mail Address:* Carnegie Institution, 5241 Broad Branch Road, Washington 15, D. C.
Council:	*Dr. L. R. Blinks* (2-year term), Hopkins Marine Station, Pacific Grove, California.
Council:	*Dr. D. E. S. Brown* (2-year term), Department of Zoology, University of Michigan, Ann Arbor, Michigan.
Council:	*Dr. Aurin M. Chase* (1 additional year), Department of Biology, Princeton University, Princeton, New Jersey.
Council:	*Dr. David R. Goddard* (1 additional year), Department of Botany, University of Pennsylvania, Philadelphia, Pa.

Society Representative on the Governing Board of the American Institute of Biological Sciences: *Dr. Harold F. Blum* (1 additional year), Department of Biology, Princeton University, Princeton, New Jersey.

NEW MEMBERS: All ten nominated candidates were elected by vote of the Society as a whole. Together with four resignations and four deaths the membership total is now 218.

TREASURER'S REPORT: In accordance with the Constitution, the books of the Society have been set up on the basis of a fiscal year ending the 30th of June, 1952. The balance of the Society was then $356.40. Since there had been no further receipts or expenditures since that date the treasury contained the same balance at the time of the meeting. It should be noted that the major expenditure was for annual dues to the AIBS, which amounted to $121.00. Receipts for dues during 10 months amounted to $256.00 including $46.00 prepaid dues. Three members of the Society have not yet remitted their current dues.

REGIONAL MEETING AT PACIFIC GROVE, MAY 10 AND 11, 1952: A successful regional meeting of the Society was organized by President Blinks at Pacific Grove during the summer. He urged that the other members of the Society plan small regional meetings in their own localities.

REGIONAL MEETING AT ST. LOUIS UNIVERSITY, DECEMBER 29, 1952: Thanks of the Society are extended to Dr. Basil J. Luyet for organizing a regional meeting, cosponsored by the A. A. A. S., on "Survival of Frozen Tissues: Basic Problems, Surgical Applications and Survival of Frozen Tumors."

MEETINGS FOR NEXT YEAR: It was announced that the Council has decided to hold next year's meeting at the Marine Biological Laboratory, Woods Hole, Massachusetts, on or about September 7 and 8, 1953. It is the Society's intention that this meeting be held immediately after the XIX International Physiological Congress, at Montreal, Canada (August 31 to September 4, 1953), and that a program of special interest both to our own members and to foreign visitors be planned. This met with general approval. Our members are urged to submit to the President any suggestions in regard to the program.

The sincere thanks of the Society were extended to Dr. Donald R. Griffin who generously consented to act as the local Society Representative to the AIBS meetings. There being no further business, the meeting was adjourned for the annual Society cocktail party, held at the Statler Inn.

New copies of the amended Constitution and bills for 1952–1953 dues have been mailed to members.

WILLIAM R. DURYEE
Secretary-Treasurer

A Symposium on Contributions of Comparative Physiology to General Physiology

The program was organized by Dr. Arthur C. Giese of Stanford University (Hopkins Marine Station)

1. Contributions from a Comparative Physiological Study of Microorganisms. R. E. HUNGATE, *Department of Bacteriology, Washington State College, Pullman, Washington.*

The physiology of microorganisms is uniquely important to general physiology because of the many metabolic reactions not found in higher forms. Among these one of the more important is the ability to meet energy requirements in the absence of oxygen. An analysis of the basic plan of microbial fermentations provides an explanation for the widespread use and occurrence of carbohydrates.

Studies on cellulose utilization by microorganisms in termites and in cattle show how their anaerobic processes are related to the food requirements of the host. The carbohydrate-nitrogen ratios of these forms and their efficiencies in utilizing nitrogen provide some interesting comparisons.

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2. Contributions from the Study of Algae to General Physiology. L. R. BLINKS, *Hopkins Marine Station, Pacific Grove, California.*

The algae have long been attractive to physiologists because of their simple structure, and often isolated cells, sometimes of gigantic size. Particularly favored thereby have been studies of permeability, salt accumulation and bio-electric phenomena. The genera *Spirogyra, Laminaria, Valonia, Halicystis, Nitella* and *Chara* need only be mentioned to indicate the extent of these contributions. Some of these will be briefly summarized.

For photosynthetic studies, ease of culturing uniform populations, absence of complications due to stomata, gas spaces and translocation, the algae have again been favored. Practically all the studies of quantum efficiency, induction phenomena, flashing light effects, and photosynthetic products have been done with algae—mostly *Chlorella*, but also diatoms and a variety of other blue-green, green, brown, and red algae. The algae are especially rich in so-called accessory pigments, whose role has been chiefly assayed in such plants. Fucoxanthin, phycocyanin, and phycoerythrin are outstanding in this regard. Rather marked lability of chlorophyll concentration and function have also been found in algae, contributing to an understanding of energy transfer. Fluorescence and a very faint after-luminescence have also been studied chiefly in algae.

Respiration and fermentation on the other hand have been little studied in algae, though some interesting oddities of metabolism have in recent years been investigated—especially the interrelations of mannitol and carbohydrates.

The physiological control of morphogenesis has also been aided by simplicity of algal structure: one of the most exhaustive studies of such effects was carried out with

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Fucus. *Acetabularia* and *Chlamydomonas* are other familiar examples, with *Bryopsis*, a fairly complicated coenocyte, now beginning to receive attention.

The validity of extending results of algal studies to other cells—in other words to *general* physiology—will be briefly discussed, with a word or two of caution.

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3. Comparative Study of Plant Metabolism. D. R. GODDARD, *Botanical Laboratory, University of Pennsylvania.*

(No abstract received)

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4. Contributions to General Physiology from the Study of Osmoregulation. C. L. PROSSER, *Department of Physiology, University of Illinois.*

The study of osmoregulation contributes to two general areas of biological theory. First, it defines the limits and permits some predictions of evolutionary trends regarding migrations of species from one salinity to another and between aquatic and terrestrial habitats. Animal life is restricted to the limits of 0.05 to 1.02 osmolar internal concentration. Given species show more restricted genotypic limits and individuals have still narrower phenotypic limits. It is probable that survival limits for osmoadjusters can vary more with acclimatization than for osmoregulators. Osmoadjusters are limited to sea water or brackish water, hypo-osmotic regulators can live on land or in salt lakes and hyperosmotic regulators show varying degrees of success in brackish and fresh water. It is predicted, on the basis of limited studies of fish and crustaceans, that tests of physiological adaptation to osmotic stress would reveal numerous genetic races within species, some with preadaptive capacities.

A second area of contribution concerns cellular mechanism of osmoregulation. The mechanisms of osmoregulation include: (1) restrictions on permeability to water and salts, (2) variation in fluid excretion, and (3) active transport of salts into or out of the regulating animal. Active transport of water is indicated in some animals and high selectivity of ionic pumps is required. All indirect evidence points to specific carriers for active transport but no such enzymes have been isolated.

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5. Persistent Rhythmicity in Animals. F. A. BROWN, *Department of Zoology, Northwestern University.*

Among animals and plants there are innumerable instances where biological activities are known which exhibit a rhythmicity correlated with cosmic events. Among the best known of these are diurnal rhythms. In many instances the diurnal rhythms and, very rarely, the tidal rhythms, have been reported to continue to obtain under conditions constant with respect to temperature, illumination, humidity, and all other factors known to influence organisms. Such rhythms are the persistent ones which are considered in this report.

The fiddler crab, *Uca pugnax*, shows a striking diurnal rhythm of migration of the pigment of its melanophores which persists without reduction or loss of precise synchrony with solar day-night for at least several weeks under constant laboratory conditions. The animals become dark in color by day and light by night. The frequency of the rhythm is uninfluenced by temperature within the range of 6 to 26° C.; it is inhibited at temperatures near 0° C. and by this means may be made to alter permanently the phases of its dark and light periods with respect to solar day-night without affecting its 24-hour frequency. By light to dark changes at sensitive times in the cycle the phases may also be altered with respect to solar day-night. Outward evidences of the rhythm may be inhibited by constant bright illumination only to reappear with accurate 24-hour frequency when the animals are removed to low light or to darkness. Altering the relative lengths of the light and dark periods in a 24-hour

cycle produces persistent corresponding shifts in the form of the rhythm but it is impossible experimentally to impose on the animals a persistent rhythm of a frequency other than 24 hours. Other characteristics of the behavior of the rhythm have led to the conclusion that two centers of diurnal rhythmicity in the crabs are involved.

In addition to the accurate diurnal rhythm, *Uca pugnax* also possesses a persistent tidal rhythm in its melanophore activity; there is a rhythm of 12.4 hours which results in augmentation of the dispersed state of the melanin such that maximum augmentation occurs about an hour before the time of low tide in its native habitat. The combination of the diurnal and tidal rhythm results in an accurate 14.8-day cycle of pigmentary change which persists under constant laboratory conditions. This is the period required for the two rhythms to pass from one time of synchrony to the next. Every second period of synchrony is a lunar cycle. These results permit interesting speculation as to the roles of peristent tidal and diurnal rhythms in the regulation of events of longer frequency such as lunar and annual reproductive cycles, and in the time sense of organisms.

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6. Contributions from Studies of Diverse Response Mechanisms in Animals. T. H. Bullock, *Department of Zoology, University of California, Los Angeles.*

Several cases are reviewed, chosen from recent studies of receptors, effectors and nervous elements, in which comparative physiology appears likely to affect previous concepts by extending significantly the range of expression of the general phenomenon or by adding degrees of freedom to the expression of the general phenomenon.

Evidence is accumulating that some poikilotherms possess an absolute in addition to relative or adapting temperature detection. The mechanism of detection is unknown but several sense organs capable of nonadapting temperature response are described. Non-nervous detection is possible but unknown. Also of general physiologic interest is the fact that this detection is apparently linked sometimes with means for compensating, in the rates of cellular events, for temperature change. These homeostatic mechanisms for metabolic and other rates are probably multiple, partly local and cellular, partly in pacemaker organs and reach equilibrium at unequal rates after imposition of temperature stress, as manifested by various functions (heart beat, oxygen uptake, movements, lethal limits, Q_{10}, size dependence of metabolism).

There have arisen in certain cases (facial pit of pit vipers) sensitive receptors for the long-wave-length radiation emitted by objects only a few degrees above the temperature of the surroundings. At present both nonspecific absorption and specific absorption mechanisms for this detection appear to be at the margin of credibility but neither can be ruled out.

The insect eyes with high temporal resolution and without light adaptation are discussed. Autrum's hypothesis suggests a centrifugal, nervous control of primary receptors, an electrical process supposed to inhibit secondary reactions of lumirhodopsin, permitting rapid regeneration.

Two muscle systems are reviewed in which independence of muscle action current and contraction, indirect central control and the large role of peripheral factors in determining time constants are emphasized. These are the adductors of Mytilus (Lowy) and the flight muscles of dipterans (Pringle, Roeder, Boettiger).

A general principle, proposed by Katsuki, states that whenever thin and thick fibers serve end organs for the same sensory modality, the former generally supply those end organs having lower threshold, slower adaptation, greater repetitiousness, lower maximum frequency, thus matching the properties of the fiber. Thick fibers are more discriminating and phasic. There appear to be only one or two partial exceptions. Since some of the available cases are receptors with primary sensory neurons, some with secondary sense cells, it is suggested that the properties of the afferent discharge are determined not by these cells but by the nerve fiber terminals. Among other cases in point, recent experiments on the statcyst of spiny lobsters are reported.

Abstracts of Contributed Papers

September 10, 1952

1. The Osmotic Behavior of the Giant Mitochondria of Insects. MARY I. WATANABE and C. M. WILLIAMS, *U. S. Army Quartermaster Research and Development Laboratories, and Harvard University.*

The spherical, interfibrillar sarcosomes of the flight muscles of dipterous and hymenopterous insects have previously been identified as the "giant" mitochondria of this extraordinary tissue. Their diameter (1 to 6 μ) is sufficiently large to permit phase-contrast microscopic observations of their osmotic behavior in the presence of various media.

Each sarcosome consists of a gelatinous matrix, devoid of Brownian motion, and surrounded by a distinct membrane. The latter is disrupted by freezing and thawing, by temperatures above 40° C., by the vapors of fat solvents, by the addition of digitonin and other surface-active agents, and by exposure to ultrasonics; the mitochondrial contents are then released as minute granules.

The sarcosomal membrane is permeable to water, electrolytes, glycerol, urea, ATP, intermediates of Krebs tricarboxylic acid cycle, pentoses, and phosphorylated hexoses. But it is essentially impermeable to disaccharides, trisaccharides, protein, and non-phosphorylated hexoses. Substances that penetrate the membrane exert only transient osmotic effects. The osmotic entry of water then causes a pronounced swelling of the sarcosome. The contents are frequently extruded, leaving the empty membrane as a discrete, swollen, spherical "ghost." The osmotic behavior of the sarcosomes appears to depend on the Donnan equilibrium. The normal architecture is best preserved in solutions containing both protein and electrolytes; *e.g.*, 2.5% crystalline bovine plasma albumin in 0.16 M potassium phosphate buffer at *p*H 7.0.

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2. Observations on Certain Properties of the Rat Liver Nuclear Envelope. N. G. ANDERSON, *Biology Division, Oak Ridge National Laboratory.*

Experiments are described which suggest that the isolated rat liver cell nucleus is permeable to high molecular weight substances including proteins and highly polymerized nucleic acids. A number of enzymes including DNAase and RNAase have been found to penetrate it rapidly and to produce the characteristic effects described. Under a number of experimental conditions it has been found possible to induce reversibly the formation of protein-impermeable blebs on the surface of isolated nuclei. The view is presented that the envelope of the intact nucleus is a meshwork-like structure, composed chiefly of tangentially oriented fibrils, with an associated lipid or lipoprotein component which may be induced to dissociate from the fibrils to form a continuous sheet. It is suggested that this mechanism may function to produce cyclic changes in nuclear permeability during cell division. The relation of these findings to certain aspects of nucleocytoplasmic interactions will be discussed. A method for preparing isolated nuclear envelopes from rat liver cells will be described.

3. Observations on the Kinetics of Sodium Extrusion from Isolated Frog Muscle. H. B. STEINBACH, *Department of Zoology, University of Minnesota.*

Using methods previously described (Steinbach, Am. J. Physiol. v. 167, p. 284) the effects of various factors on the rate of sodium extrusion from sodium-enriched frog sartorius muscles have been studied. It has been found that during the first 30 minutes in potassium-containing recovery solutions, the amount of sodium extruded from the fibers is roughly linear with time, the new equilibrium being about 90 percent complete in one hour. A minimum average initial rate for the process is 40 mmols Na per kg. fiber per hour. This fast process of sodium extrusion is highly sensitive to temperature, the rate at 2° C. being roughly one-seventh that at 22° C.

When the initial sodium concentration of the sodium-enriched muscles is greater than about 60 mmols per kg. fiber, the initial rate of sodium extrusion is independent of the internal sodium concentration. At lower intrafibral sodium concentrations, sodium extrusion is a direct function of internal sodium concentration.

When the potassium concentration of the external recovery solution is greater than 20 mmols per liter, the initial rate of sodium extrusion is independent of external potassium concentration. With lower external potassium concentrations, sodium extrusion is dependent on external potassium concentrations.

The data are interpreted as indicating that sodium extrusion involves combination of the metal with some substance, presumably in the external limiting layers of the fibers, to form a specific carrier compound. The rate of sodium extrusion depends, among other things, on temperature, internal sodium concentration, internal concentration of the combining substance and rate of inward leakage of potassium to compensate for the positive charges moved outward during sodium extrusion.

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4. Experimental Shift in Flux Equilibrium of Hydrogen Donor Systems and the Mechanism of Rebound in Bio-electrical Potential. E. J. LUND and W. E. NORRIS, JR., *University of Texas.*

During temporary removal of onion root tips from oxygen, hydrogen donor concentration increases, as shown by the transient increase in methylene blue reduction (respiratory rebound). This rebound is greater in the apex meristem than in the older tissues, thus corresponding in time and distribution to electrical field rebound after temporary removal of oxygen, as previously shown.

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5. The Contact Theory of Bio-electricity and Its Application to the Theory of Nerve Function. R. H. BEUTNER, *West Massachusetts School of Pharmacy.* (Presented by Title)

The Contact Theory postulates that electromotive forces arise in living tissue in the same manner as in the well known inorganic battery systems. According to established electrochemical principles, the electromotive force of these battery systems is made up of two electric double layers or "contact potential differences" as we may call them, located at the metallic electrodes. In living tissues contact potential differences arise where lipoids or lipoproteins are in contact with aqueous solutions. The problem is to determine which contacting substances can produce potential differences in tissues. An extensive study of these potential differences has been carried out at the contact of various aqueous solutions and water-immiscible fluids (R. Beutner, "Bio-electricity" in *Medical Physics*, 1944). Their physico-chemical nature was thoroughly elucidated by this work, but it remained to determine their physiological significance. For the general physiologist, the most important property of these potential differences is their negative variation when contacted by acetylcholine or other neurohormones (T. C. Barnes and R. Beutner, 1941 ff.). In all these experiments

the lipoid was "represented" by such body-foreign substances as phenol derivatives, so that a physiological application was somewhat uncertain. More significant results were obtained when it became possible, through an improvement of the technique, to use real brain lipids, and contact these with neurohormones (R. Beutner and L. Williams, Federation Proc., *10*, 1952). Only freshly extracted brain lipids were found to be active. More recent studies showed that extracted brain lipids are not only inactive after decomposing but actually antagonize the electrical effect of normal lipids when added to them. If we assume that the functional activity of nerve can be impaired and antagonized by a chemical decomposition of its lipids, we could account on the basis of these recent findings for the inhibiting influence exerted by diseased parts of the brain, and possibly for the clinical results obtained after prefrontal lobotomy.

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6. Relation of Na Uptake and K Rejection to Membrane Potential in Isolated Surviving Frog Skin. E. G. Huf and Joyce Wills, *Medical College of Virginia.*

Isolated surviving frog skin, when bathed with the same kind of diluted Ringer's solution on both sides, shows a negative correlation between net active salt uptake by the epithelium and spontaneous skin potential. Average values of 0.15 to 0.86 μEq $\times$ hr.$^{-1}$ $\times$ cm.$^{-2}$ were measured and correlated to average skin potentials ranging from 107 to 25 MV. Sodium uptake exceeded chloride uptake by about the same amount, irrespective of the height of skin potential. The same skins which exhibited a negative correlation between net uptake of sodium chloride and skin potential showed a positive correlation between net potassium rejection from the epithelium and skin potential, for voltages above 30 to 40 MV. In skins with voltages lower than this, potassium ions were taken up rather than rejected. Average values for rejection of $+11.8$ to -0.8 centiμEq $\times$ hr.$^{-1}$ $\times$ cm.$^{-2}$ were measured. Net fluid uptake, associated with active uptake of sodium chloride, was small and occurred in the direction of the salt uptake. No dependence of net fluid uptake upon skin potential was observed. Skins of winter frogs, pretreated with a commercial purified ACTH preparation, were less active than their respective controls with regard to uptake of sodium chloride. Rejection of potassium was the same in treated and untreated skins. Posterior pituitary factors, as possible contaminants, did not account for the effect of the ACTH preparation. DOCA, DOC and cortisone did not alter the normal correlation referred to above. In interpreting the experimental results on theoretical grounds, it is suggested (*a*) that in normal skin, it is the variation in the electric conductance in skin of chloride ions which essentially, although not exclusively, determines the rate of net uptake of sodium chloride; (*b*) that a factor in the ACTH preparation used, possibly ACTH itself, may have lowered the electric conductance in skin of sodium ions either truly or apparently; (*c*) that potassium ions are treated by the skin primarily as passive ions. There is some indication that potassium ions are also actively taken up by the epithelium of skin.

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7. On the Tendency of Glycerol-Impregnated Tissue to Supercooling. B. J. Luyet, *Institute of Biophysics, St. Louis University.*

The impregnation of animal tissues with glycerol renders them resistant to injury by freezing, enabling them to produce abundant growth in tissue culture. In a study of the factors involved in this protective action, we investigated, first, the possibility that glycerol imparts to the water of the tissues its tendency to supercool. Pieces of chick-embryo heart measuring about one cubic millimeter, immersed in 30% or 60% glycerol solution or in pure glycerol for lengths of time varying from 30 seconds to 4 minutes and afterwards exposed in cooling baths of temperatures to $-20°$, $-30°$ and $-40°$ C. did not begin to freeze until some time after the untreated control pieces. The delay was longer after immersion in higher concentrations of glycerol and after

longer times of immersion. After a 4-minute immersion in pure glycerol practically all the pieces remained supercooled for at least 5 minutes in baths of −20° and −30°. Thus glycerol exerts some impeding action on the formation of crystallization centers in the tissues impregnated with it.

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8. Minimal Exposures Needed to Acclimatize Rats to Cold Atmospheres. M. J. FREGLY, *Department of Physiology, University of Rochester.*

By means of a simple cooling test it is possible to ascertain how long it takes for cold acclimatization to develop and how long it persists.

The cooling test consisted in taping a rat to a board by its four legs. A thermocouple was placed in the colon and the temperature changes there were registered on a recording potentiometer from many individuals simultaneously. Unacclimatized and acclimatized rats were then placed in an atmosphere of 5° C. until each cooled to 18° C. The rates of colonic cooling were then compared.

Individuals living in cages within the cold-box air of 5° C. for 2 days showed the same rate of cooling as those never before exposed. But after sojourning 4 to 10 days, cooling was 35% slower. The slower cooling then persisted at any time during two weeks of no exposure; thereafter it disappeared.

Acclimatization was also conferred by virtue of the cooling test alone in three successive exposures on alternate days. The acclimatization thus conferred did not differ from that of rats exposed to cold continuously for 5 days.

Rats which had lived in 5° C. air for 2 or more days lost their righting reflexes significantly more quickly than controls when taken for a few minutes to 39,000 feet altitude. Loss of righting reflexes occurred more quickly with increasing time spent in the cold so that this simple test may also be used as a criterion of acclimatization to cold. Hence, exposure to cold made the rats less able to withstand altitude.

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9. Differences in P^{32} Turnover Between Two Related Species of Drosophila. R. C. KING, *Brookhaven National Laboratory.*

Males and females of *Drosophila melanogaster* and *D. simulans* were allowed to feed upon a P^{32}-containing medium for their entire larval period and for a portion of their adult life. The adults were then transferred to P^{32}-free medium and the rate of loss of radioactivity determined for a ten-day interval. The resulting curves show a decided break between the fourth and fifth day when plotted semilogarithmically. The curves may be interpreted as resulting from two phase systems described by the formula $y=i_1e^{K_1t}+i_2e^{K_2t}$. For *melanogaster* males $i_1=0.51$, $K_1=-0.4158$, $i_2=0.49$, $K_2=-0.0800$; females $i_1=0.36$, $K_1=-0.4620$, $i_2=0.49$, $K_2=-0.0866$. For *simulans* males $i_1=0.55$, $K_1=-0.3780$, $i_2=0.42$, $K_2=-0.0408$; females $i_1=0.43$, $K_1=-0.5198$, $i_2=0.55$, $K_2=-0.0990$. Since the absolute amount of phosphorus in the flies remains constant, the values determined represent rates of total phosphorus turnover. The half-time for total phosphorus turnover for *simulans* males=3¼ days, females =2⅔ days; *melanogaster* males =3 days, females =2½ days. Thus males lose their phosphorus more slowly than females, while for a given sex *simulans* adults lose phosphorus more slowly than *melanogaster*. All four groups have fast phases of similar half-time. The slow phase of *simulans* males is significantly slower than any of the other slow phases. In both species males excrete significantly more phosphorus by the fast phase than do females. Females of *melanogaster* excrete 15 percent of their phosphorus by way of a very fast phase of unknown half-time.

10. The pO_2–Q_{O_2} Relation in the Phormia Larva. JOHN B. BUCK and MARGARET L. KEISTER, *National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, Federal Security Agency, Bethesda, Maryland.*

Oxygen uptake was measured in larvae made hypoxic either by exposure to 1, 2, 5 or 10% oxygen or by ligating off one or both pairs of spiracles; in larvae with metabolism stimulated by DDT or by temperatures above 25° C.; and in larvae at 10°, 15° and 20° C.

It is concluded that (1) in doubly-ligated larvae at 25° the internal pO_2 is zero, and respiration is limited by oxygen at least at 760 mm. and below; (2) in larvae with the posterior spiracles ligated the respiration is oxygen-limited at 15° and above; (3) in larvae with the posterior spiracles ligated and metabolism stimulated with DDT, oxygen is limiting to respiration at temperatures above about 5°; (4) the internal pO_2 changes inversely with degree of hypoxia, but not linearly; (5) the temperature results can be interpreted as favoring the view that hypoxia consists of a rather general tissue oxygen deficiency, as opposed to the view that it involves local regions of total anoxia.

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11. Postradiation Loss of ATP from *Escherichia coli* B/r. D. BILLEN, B. L. STREHLER, G. E. STAPLETON, and E. BRIGHAM, *Oak Ridge National Laboratory.*

A study of the effects of X rays on adenosinetriphosphate (ATP) metabolism of *Escherichia coli* B/r has been initiated because of the importance of this compound in the anabolic reactions of living cells. ATP was assayed by measurement of light emitted when this compound is incubated with the firefly luminescent system.

It has been found that *E. coli* B/r, although exposed to 60,000 r, (99.95% kill) retain the ability to form ATP if incubated at 37° C. in nutrient broth containing 1% glucose. However, upon separation of the cells by centrifugation from the incubating medium, it was found that the irradiated cells contained decreasing quantities of ATP while the supernatant showed increasing ATP content as the duration of the incubation period was increased. The control cells did not show this loss of ATP from the cells to the medium. The suspending medium constituents as well as the temperature of incubation affected the degree of ATP loss. The loss of ATP was also found to be dose-dependent since increasing doses of X rays cause increased loss from the cells. From the available evidence the loss of ATP appears to be due to a permeability change, or possibly some subtle form of lysis, since no gross lysis was evident.

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12. Extension of the Life Span of Spermatozoa of Marine Invertebrates by Means of Heavy-Metal-Chelating Agents. A. TYLER, *Division of Biology, California Institute of Technology.*

Previous work showed that the duration of motility and of fertilizable life of spermatozoa of various invertebrates could be considerably extended by the addition of any one of a number of amino acids to the suspension medium. It was also demonstrated that the added amino acid is not utilized metabolically. There is, however, a marked metabolic effect in that the initial great increase in respiratory rate, that occurs when the spermatozoa are diluted, is inhibited in the presence of the amino acid and respiration is maintained at a low, relatively constant level after that of the controls has dropped practically to zero. It was shown that the normal early death of these cells, when suspended in sea water, is not due to "starvation." The added amino acid enables the spermatozoa to utilize more completely their endogenous substrate. Another effect of the added amino acid, that occurs without its being utilized metabolically, is to enable the spermatozoa to survive anaerobically for a considerable period whereas normally they quickly become motionless and die in absence of oxygen. It is known that amino acids form sparingly ionized complexes

with ions of heavy metals. This, along with the evidence of nonutilization, suggested that they act by binding certain heavy metal ions ordinarily present in the dilution medium in amounts which are barely detectable but sufficient to cause a rapid progressive "intoxication" of the spermatozoa. Tests with other kinds of chelating agents have supported this view. The extension of life span and other effects of the amino acids have been obtained with such chelating agents as diethyldithiocarbamic acid, α-benzoinoxime ("cupron"), 8-hydroxyquinoline ("oxine") and ethylenediamine-tetracetic acid ("Versene"). The experiments do not, as yet, permit exact identification of the particular heavy metal ions involved but it appears likely that copper and zinc are the principal ones.

Announcement

Report of the Joint Committee on Reporting Cancer End Results [1]

A Standardized Method for Reporting Cancer End Results

The lack of uniformity in reporting cancer end results has been a source of worry to clinicians and research workers seeking to evaluate therapy from published reports. In order to overcome this difficulty, the Joint Committee on Reporting Cancer End Results [1] was established to recommend rules and forms aimed at eliminating the major causes of confusion. The Committee was composed of representatives of the American College of Surgeons, the College of American Pathologists, the American College of Radiology and the American Cancer Society with the addition of one member-at-large from the National Cancer Institute. The following report was adopted by the Committee.

The purpose of any statistical analysis is to obtain reliable answers to certain specific questions. Therefore, the analytic method of choice depends upon the nature of the questions as well as upon the data available and it is frequently necessary to employ several different methods in order to answer various questions. For this reason, no one method of analyzing cancer end results can be recommended as a solution to all the problems which may arise. Nevertheless it is possible to outline certain basic principles and to suggest a simple standard type of analysis applicable to most sets of data on this subject. More detailed supplementary analyses would then depend upon the exact problem which the investigator seeks to elucidate.

The following are among the most common of the numerous reasons for reporting cancer end results:

(1) Comparison of the effectiveness of alternative methods of treatment, *a*) in achieving permanent cures, *b*) in extending the life of the patient whether or not permanent cures are achieved, *c*) in recurrent cases of cancer, *d*) in the relief of suffering in incurable cases of cancer.

(2) Analysis of the influence on the course of the disease of such factors as *a*) histologic type, *b*) size and extent of the lesion, *c*) evidence of metastasis, *d*) duration of symptoms, *e*) age and condition of patient.

While there are various perfectly correct methods by which these comparisons may be made, it is obvious that two different series of cases can be compared only if the same method of analysis is used for both. For example, the effectiveness of a certain treatment used by one doctor can be compared with the effectiveness of an alternative method of treatment used by another doctor only if the end results are presented in exactly the same way. Thus a standardized method of reporting is essential when the purpose of reporting is to make such comparison possible. This in no way limits the freedom of the investigator to make additional analyses by other or more detailed methods when desirable.

A great deal of confusion exists in the reporting of cancer end results. Widely different cure rates are published for what appears to be the same type of cancer and it is frequently impossible to tell whether the differences are due to variations in the method of treatment or whether they are due to variations in the method of reporting the results. The principal reasons for this confusion seem to be:

[1] Membership on the Joint Committee on Reporting Cancer End Results—Representing the American College of Surgeons: Ernest M. Daland, M. D., Grantley W. Taylor, M. D., Murray M. Copeland, M. D. Representing the College of American Pathologists: B. Earl Clarke, M. D., Maxwell J. Fein, M. D., Theodore J. Curphey, M. D. Representing the American College of Radiology: Donald S. Childs, M. D. (*Chairman of Committee*) Harold W. Jacox, M. D., Frederick W. O'Brien, M. D. Representing the American Cancer Society: Alton Ochsner, M. D., E. B. Wilson, Ph. D., E. Cuyler Hammond, Sc. D. (*Secretary of Committee*). Member-at-large: Harold F. Dorn, Ph. D., of the National Cancer Institute, U. S. Public Health Service.

(*a*) *Selection of cases.* The result of therapy depends in no small measure upon the condition of the patient at the time treatment is initiated, and there are great variations in the type of patients coming to the attention of different doctors. For example, some doctors see a preponderance of advanced or recurrent referred cases while other doctors see a preponderance of relatively early cases and refer their advanced cases for treatment elsewhere. Aside from this there is frequently a selection in the cases reported in the literature. Some authors present only "hopeful" cases, some present all cases treated (whether hopeful or not), and some present all cases seen (including those not treated).

(*b*) *Proof of diagnosis.* Some authors present only cancer cases with microscopic confirmation of the diagnosis, some include cases without microscopic confirmation of the diagnosis, and still others simply make no mention of the method of diagnosis.

(*c*) *Inadequate follow-up.* Comparatively few doctors or hospitals are able to obtain a five-year follow-up on every diagnosed case of cancer. Survival rates are usually based only upon those cases which were followed, but frequently no mention is made of the number of cases which were lost to follow-up. This also can make a considerable difference in survival rates since it cannot be assumed that untraced cases had the same history as traced cases. From our observations, it appears that the proportion of cases followed for five years varies from 100 percent down to a very low percentage indeed.

(*d*) *Cure rates vs. survival rates.* "Five-year cancer cures" are distinguished from "five-year cancer surviviors" by the fact that the former are cases which are alive and apparently free of cancer five years after diagnosis, while the latter are all cases alive five years after diagnosis including cases alive but with evidence of cancer. While both five-year cure rates and five-year survival rates are useful (though for somewhat different purposes), serious error results from mislabeling or failure to specify clearly which of the two is meant. Another difficulty which arises in this matter is the fact that in reporting five-year cure rates there has been no uniform method of handling cases which die of causes other than cancer before the end of the five-year period.

The rules and standard forms adopted by the Committee are given at the end of this report. They are designed for the presentation of minimum basic information in a uniform manner. It should be emphasized that the investigator is expected to give additional information in supplementary tables and in the text. This will give the reader the necessary data to make analyses most suitable for the purpose he has in mind. For example, the reader will be able to compute five-year cure rates or five-year survival rates as he sees fit. In some instances, an author may wish to supplement his report with information on yearly results up to and beyond the five-year period.

Certain problems in connection with the use of the standard form should be clarified. In reporting on a series of cases, a form should first be filled out including every case seen of the particular site or type under consideration. The series should be precisely identified in terms of site, source of cases, and time-period covered. Supplementary forms may then be filled out for selected subgroups classified according to such factors as stage, grade, histologic type or method of treatment.

The "General Summary" section at the top of the form need only be filled out for the over-all series of cases. It is included to insure that all cases are reported without selective bias. The total experience is divided into four mutually exclusive categories: Section A, Section B, Other (a), and Other (b). Those cases classified as "Other" appear only in the summary; whether or not these cases had been treated previously is immaterial and no follow-up information is required on them.

Rules for Reporting Five-year End Results of Persons With Malignant Neoplastic Diseases

1. Include *all* cases diagnosed as having a malignant neoplasm, whether treated or not. Whenever possible, all cancers should be classified according to the site of origin.

2. Cases reported should be divided into two sections, A and B, as defined below:

Section A: Cases not previously treated.—The duration of these cases is measured from the date of the original diagnosis of this cancer.

Section B: Cases previously treated.—The duration of these cases is measured from the beginning of subsequent treatment.

The total number of cases in Section A and the total number of cases in Section B should always be reported. However, if the investigator so desires, he may report the full details for one of these two sections only. It is generally desirable to report every case from the date of original diagnosis until the end of the 5-year period. If this is done, then all cases will fall in Section A. The detailed tabulation of Section B cases is designed primarily for those investigators who wish to report on the efficacy of treatment in recurrent cases of cancer.

3. Include only cases with an elapsed duration of at least 5 years. Report the status of each patient on the fifth anniversary. Each patient should be examined at least yearly.

4. The condition of patients classed as alive and free from cancer must be established through examination by a physician at the end of the 5-year period or at a later date.

5. The percentage of cases with a microscopically confirmed diagnosis is a measure of the accuracy of diagnosis. This percentage should be as high as possible; however, cases without microscopically confirmed diagnosis should be included but shown separately.

6. Whenever possible, an autopsy should be performed. A high degree of accuracy requires that autopsies be performed at least on all those patients who die before the end of the 5-year period.

7. The percentage of cases untraced for the full 5 years should be as close to zero as possible. If it is greater than ten percent, the report will be of doubtful validity.

8. In reporting on cancer of a given primary site, patients will be classified according to the status of that disease. If a new primary histologically proven cancer develops, it should be considered as an intercurrent disease even though it may cause the death of the patient.

9. It is most important that the report should include all the basic data shown on the accompanying forms. This is considered to be the minimum amount of information needed for a useful report; many investigators will wish to give additional information in supplementary tables and in the text. It is left to the discretion of the investigator whether he wishes to compute 5-year survivor rates or 5-year cure rates or both.

10. The following form is recommended for use in tabulating end results.

October 1951

A Report of 5-year End Results

Cancer of .....

This series consists of all patients with cancer of the ....., both early and advanced, applying to ..... during the period ..... to .....

General summary		Number of cases
Total experience—All patients applying		
Section A..	Cases not previously treated	
Section B..	Cases previously treated	
Other.....	a. Applied after treatment elsewhere, no evidence of cancer on admission or thereafter	
	b. Consultation only, no treatment requested	

Section A—Cases not previously treated

Results at end of 5 years		Number of cases		
		With microscopic proof	Without microscopic proof	Total
Group I	c. Refused proffered treatment			
	d. Untraced for full 5 years without recurrence at last examination			
Group II	e. Dead within 5 years of other causes without recurrence of cancer			
Group III	f. Dead, cancer present or died of complications of treatment			
	g. Dead—presence of cancer unknown			
	h. Living with cancer present at 5 years			
	i. Living with condition unknown at 5 years			
	j. Untraced full 5 years with cancer at last examination			
	k. Untraced full 5 years, not classifiable in "d" or "j"			
Group IV	l. Living, continously free of cancer, verified by medical examination at 5 years			
	m. Living, apparently free of cancer, not verified by medical examination at 5 years			
	n. Living, successfully treated for recurrence, free of cancer at 5 years			
Total				

Section B—Cases previously treated

Results at end of 5 years		Number of cases		
		With microscopic proof	Without microscopic proof	Total
	c. Refused proffered treatment			
Group I	d. Untraced for full 5 years without recurrence at last examination			
Group II	e. Dead within 5 years of other causes without recurrence of cancer			
Group III	f. Dead, cancer present or died of complications of treatment			
	g. Dead—presence of cancer unknown			
	h. Living with cancer present at 5 years			
	i. Living with condition unknown at 5 years			
	j. Untraced full 5 years with cancer at last examination			
	k. Untraced full 5 years, not classifiable in "d" or "j"			
Group IV	l. Living, continuously free of cancer, verified by medical examination at 5 years			
	m. Living, apparently free of cancer, not verified by medical examination at 5 years			
	n. Living, successfully treated for recurrence, free of cancer at 5 years			
Total				

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